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Steven L. Highlander

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Commissioner for Patents
P.O. Box 1450
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Re: Serial Number 09/981,682 entitled "METHOD OF INHIBITING VIRAL INFECTION USING HMG-COA REDUCTASE INHIBITORS AND ISOPRENYLATION INHIBITORS" by Barney Graham et al.
Our ref: VBLT:003US

Commissioner:

Transmitted herewith for filing are:

1. Brief on Appeal (an original and three copies);

2. A check in the amount of $165.00 for filing an Appeal Brief; and

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Barney GRAHAM, Tara GOWER, and,
Manoj PASTEY

Serial No.: 09/981,682

Filed: October 16, 2001
For: METHOD OF INHIBITING VIRAL INFECTION USING HMG-COA REDUCTASE INHIBITORS AND ISOPRENYLATION INHIBITORS

Group Art Unit: 1617

Examiner: S.A. Jiang

Atty. Dkt. No.: VBLT:003US/SLH

APPEAL BRIEF

BOX AF
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

Appellants hereby submit an original and two copies of this Appeal Brief to the Board of Patent Appeals and Interferences in response to the Office Action dated October 2, 2003, which is due on May 23, 2004, by virtue of the Notice of Appeal filed on March 23, 2004. The fee for filing this Appeal Brief is attached hereto. If the check is inadvertently omitted, or should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed material, the Commissioner is authorized to deduct said fees from or to Fulbright & Jaworski L.L.P. Account No.: 50-1212/VBLT:003US/SLH.

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Appendix 1: Pending Claims
Appendix 2: Exhibits

A - Mazière *et al.*
B - Park *et al.*
C - Pastey *et al.*
D - Fisher *et al.*
E - Gruber *et al.*
F - US 6,573,247
G - US 6,558,923
H - Crowe et al. (C32, PTO-1129 submitted June 11, 2002)

I. Real Party in Interest

The real party in interest is the assignee, Vanderbilt University.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of the Claims

Claims 1-50 were filed with the application. Claims 10, 12 and 21-50 have been canceled. Claims 1-9, 11 and 13-20 are pending and stand appealed. A copy of the pending claims are attached as Appendix 1.

IV. Status of Amendments

Appellants submitted amendments to the claims on March 23, 2004, which amendments were not entered.

V. Summary of the Invention

The present invention relates to a method of using inhibitors of HMG-CoA reductase and isoprenylation to inhibit a cellular entry receptor for a number of viruses, including HIV-1, RSV and Parainfluenza virus-3 (PI-3). Specification at page 4, lines 3-5. In one embodiment, inhibitors of HMG-CoA reductase and/or isoprenylation are used prophylactically to prevent infection by viruses that utilize the RhoA receptor. Specification at page 4, lines 5-7. In another embodiment of the invention, one or more inhibitors of HMG-CoA reductase are administered to a subject diagnosed with an existing viral infection to prevent the spread of the infection from cell to cell within the body. Specification at page 4, lines 19-21. Other embodiments include those combining HMG-CoA reductase inhibitors and isoprenylation inhibitors. HIV, RSV and PIV are particular targets for these therapies. Specification at page 4, lines 29-31.

VI. Issues on Appeal

Are claims 1-9, 11, and 13-20 indefinite under 35 U.S.C. § 112, second paragraph?

Are claims 1 and 8 anticipated under 35 U.S.C. §102(b) by Maziere *et al.* (Maziere; Exhibit A)?

Are claims 1-9, 11, 13, 15-16, and 19-20 obvious under §103(a) over Maziere and Park *et al.* (Park; Exhibit B) in view of Pastey, Gower *et al.* (Exhibit C)?

Is claim 14 obvious under §103(a) over Maziere and Park in view of Pastey, Gower *et al.*?

Is claim 17 obvious under §103(a) over Maziere and Park in view of Pastey, Gower *et al.* and Fisher *et al.* (Fisher; Exhibit D)?

Is claim 18 obvious under §103(a) as being unpatentable over Maziere and Park in view of Pastey, Gower *et al.* and Gruber *et al.* (Gruber; Exhibit E).

VII. Grouping of the Claims

Claims 3-6 present separate issues of patentability, as discussed in detail below in Section IX.H.

VIII. Summary of the Argument

The examiner has rejected the terms "a subject," "severe combined immunodeficiency," "an inhibitor of isoprenylation that is distinct from said HMG-CoA reductase inhibitor," "a nucleoside analog composition" and "a protease inhibitor" as indefinite. Appellants submit that each of the terms to which the examiner objects are definite, and that the examiner has not established why, in light of their use in the specification, such terms are unclear.

Claims 1 and 8 are not anticipated by Maziere *et al.*, eitner inherently or expressly, for the simple reason that claim 1 recites "[a] method of inhibiting infection of a cell by a virus in a subject comprising the step of administering to said subject an inhibitor of HMG-CoA reductase." Since Maziere only teaches that the HMG-CoA reductase inhibitor, lovastatin, is capable of inhibiting HIV expression in the human H9 ***cell line***, it cannot anticipate use of HMG-Coa reductase inhibitor to prevent HIV infection of cells in a ***subject***, specifically *in vivo*. Accordingly, claims 1 and 8 clearly are not anticipated by Maziere.

Claims 1-9, 11 and 13-20 stand rejected under §103(a) as being unpatentable over Maziere *et al.* and Park *et al.* in view of Pastey, Gower *et al.*, optionally in combination with Fisher *et al.* or Gruber *et al.* The examiner has failed to make out a *prima facie* case in that the elements required for a proper obviousness rejection have not been established. First, none of the cited references discloses a method of using HMG-CoA reductase inhibitors to inhibit infection of a cell by a virus in subject *in vivo*, and thus they do not teach or suggest all of the claim limitations found in claim 1.

Second, there is no suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. At best, the examiner has advances a circuitous connection between Maziere and Pastey, Gower *et al.* through Park, and has even ignored teachings away in the references.

And third, the references fail to provide any evidence or reason to believe that inhibition of RhoA geranylgeranylation also inhibits viral expression. In fact, no mention is made at all whether inhibition of RhoA geranylgeranylation could be successful in inhibiting RSV

expression, much less inhibition of HIV-1. Thus, no person skilled in the art would have a reasonable expectation of success by combining the teachings of Park and Pastey, Crowe *et al.*, in the context of Maziere's teachings.

IX. Argument

A. Standard of Review

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Rejection Under 35 U.S.C. § 112, Second Paragraph

Claims 1-9, 11, and 13-20 stand rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for failing to point out and distinctly claim the subject matter which appellant regards as the invention. Specifically, the examiner mentions the expression "a subject," in claim 1, as not being defined in the specification, and thus can be interpreted as a single cell.[1] Appellants submit that it is entirely unreasonable to read "a subject" as anything but an intact

[1] Appellants offered an amendment after final changing this term to "patient," but the amendment was denied entry on the grounds it did not materially reduce the number of issues on appeal (this was not explained) or place the application in condition for allowance.

organism. There is no logical interpretation of this term that would justify an argument that it reads on a cell. Thus, the rejection is improper and should be withdrawn.

Next, the examiner argues that the expression "severe combined immunodeficiency" in claim 4 is indefinite because "severe" is a relative term and the expression is not defined in the specification. The examiner cannot conduct prosecution in a vacuum. The term severe combined immunodeficiency defines a particular disease state that is well known to those of skill in the art. In fact, a Google® search of this term resulted in over **25,000 hits**!!!!! There is even a website using the acronym for severe combined immunodeficiency – scid – at www.scid.net. Thus, it cannot seriously be argued that this term is indefinite. More importantly, the examiner has offered no evidence to support the rejection, and hence, the decision in *In re Gartside* mandates reversal of this rejection.

The examiner also argues that the specification does not clearly define the phrase "an inhibitor of isoprenylation that is distinct from said HMG-CoA reductase inhibitor," and that the terms "a nucleoside analog composition" and "a protease inhibitor" in claims 15 and 16, respectively, are indefinite as not being defined in the specification. Appellants once again traverse. In order for claims to satisfy the definiteness standard, the inventor need only ensure that "one skilled in the art would understand the bounds of the claim when read in light of the specification." *Miles Lab., Inc. v. Shandon Inc.*, 997 F.2d 870, 27 U.S.P.Q.2d 1123 (Fed. Cir. 1993). Claims need only "reasonably apprise those skilled in the art" as to their scope and be "as precise as the subject matter permits." *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 231 U.S.P.Q. 81 (Fed. Cir. 1986). Furthermore, "[t]he purpose of claims is not to explain technology or how it works, but to state the legal boundaries of the patent grant. A claim is not

'indefinite' simply because it is hard to understand when viewed without the benefit of the specification." *S3 Inc. v. nVIDIA Corp.*, 239 F.3d 1364, 59 U.S.P.Q.2d 1745 (Fed. Cir. 2001).

Section IV, page 17 is entitled "Inhibitors of Isoprenylation" which is clearly distinct from the previous section entitled "Inhibitors of HMG-CoA Reductase." Section IV contains a detailed description of geranylgeranyl transferase and farnesyl transferase inhibitors which are both clearly distinguishable from the previously described HMG-CoA reductase inhibitors. One skilled in the art would reasonably understand that "an inhibitor of isoprenylation that is distinct from said HMG-CoA reductase inhibitor," as read in light of the specification, refers to compounds such as geranylgeranyl transferase and farnesyl transferase inhibitors. The examiner has not even bothered to address this line of argument. As such, the record reflects the absence of evidence supporting the rejection, and that the burden remains with the examiner to establish indefiniteness.

Finally, with regard to the terms "nucleoside analog composition" and "protease inhibitor," appellants again emphasize that the definiteness standard is whether "one skilled in the art would understand the bounds of the claims." Both nucleoside analogs and protease inhibitors are well known drugs for treating viral infections such that one skilled in the art would know the meaning of those terms, and the examiner has offered no evidence to suggest that these terms are in any way indefinite.

Further, in U.S. Patent 6,573,247 (McGuigan *et al.*; Exhibit F), the term "nucleoside analog" is used in the abstract, the claims, and the specification without detailed explanation. Evidence that one skilled in the art would know what is meant by the term "protease inhibitor" can be found in U.S. Patent 6,558,923 (Paulous *et al.*; Exhibit G), where the terms "protease"

and "protease inhibitor" are used numerous times in the abstract, claims, and specification without being expressly defined in the specification. The examiner's only response is to argue that each patent is examined on its own merit. ***This misses the point entirely!!!*** The point is that these ***issued patents*** do not bother to define these terms, so why should the instant application, ***filed later than these other patents***, have to do so? Once again, the examiner has failed to shift the burden to appellants on this issue.

Accordingly, appellants respectfully request that the rejection of claims 1-9, 11 and 13-20 under 35 U.S.C. § 112, second paragraph be reversed.

C. Rejection under 35 U.S.C. § 102(b)

Claims 1 and 8 stand rejected under 35 U.S.C. §102(b) as being anticipated by Maziere *et al.* (Maziere). The examiner contends that Maziere discloses a method of inherently treating or inhibiting infection of a cell by a virus as described in claims 1 and 8. The examiner argues that because Maziere teaches that HMG-CoA reductase inhibitors are useful inhibiting HIV-1 expression in H9 human T lymphocytes they are useful in inhibiting the HIV cycle in patients. Appellants respectfully traverse, as claims 1 and 8 are not inherently nor expressly anticipated by Maziere.

As argued previously, anticipation requires that each and every element of the claimed invention be described, either expressly or inherently, in a single prior art reference. *Telemac Cellular Corp. v. Topp Telecom, Inc.*, 247 F.3d 1316, 1327, 58 U.S.P.Q.2d 1545, 1552 (Fed. Cir. 2001); *Verdegaal Bros., Inc. v. Union Oil Co.*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987). Appellants presently claim "[a] method of inhibiting infection of a cell by a virus in a subject comprising the step of administering to said ***subject*** an inhibitor of HMG-CoA

reductase" where the virus can be the HIV virus as well other various types of viruses. Emphasis added. Since Maziere only teaches that the HMG-CoA reductase inhibitor, lovastatin, is capable of inhibiting HIV expression in the human H9 ***cell line***, it cannot anticipate use of HMG-Coa reductase inhibitor to prevent HIV infection of cells in a ***subject***, specifically *in vivo*.

The examiner's only rebuttal is to argue that "treatment of a subject" reads on *in vitro* methods. Appellants cannot fathom such an interpretation based on the use of the term "subject" in common vernacular. Moreover, appellants offered a clarifying amendment where "subject" was changed to "patient," which amendment was denied entry. Regardless, this is no evidence of record to support the examiner's position that the plain meaning of the term "subject" could be twisted into reading on a cell. Accordingly, claims 1 and 8 clearly are not anticipated by Maziere. Therefore, reversal of the rejection is requested.

D. Rejection Under 35 U.S.C. § 103(a) - Maziere & Park *et al.* in view of Pastey *et al.*

Claims 1-9, 11, 13, 15-16, and 19-20 stand rejected under §103(a) as being unpatentable over Maziere and Park *et al.* (Park) in view of Pastey *et al.* (Pastey, Gower *et al.*). Maziere discloses the use of a HMG-Coa reductase inhibitors as a method of inhibiting HIV-1 expression in H9 human lymphocytes. Park teaches the use of HMG-Coa reductase inhibitors such as simvastatin and astrovastatin to inhibit the geranylgeranylation of RhoA GTPase. The examiner argues that it would have been obvious to one of ordinary skill in the art at the time of the invention, in view of the knowledge disclosed in Pastey, to employ HMG-Coa reductase inhibitors in a method of inhibiting infection of a cell by a virus such as RSV in a human, non-human, or a livestock mammal. The examiner also contends that through the teachings of Park and Pastey, one of ordinary skill in the art would have reasonably expected that HMG-CoA

reductase inhibitors would have a beneficial therapeutic effect in inhibiting infection of a cell by a virus such as RSV in a subject such as in a human, a non-human mammal, or a livestock animal. Finally, the examiner argues that one of ordinary skill in the art at the time of the invention was made would have been motivated to employ HMG-CoA reductase inhibitors in combination with a nucleoside analog composition in a method of inhibiting infection of a cell by a virus such as RSV in a human, non-human, or a livestock mammal. Appellants respectfully traverse.

In order to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success; and (3) the prior art reference (or references when combined) must teach or suggest all the claim limitations. *Manual of Patent Examining Procedure* §2142. See also *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed Cir. 1991) (emphasizing that the teaching or suggestion to make the claimed combination and the reasonable expectation of success must be both found in the prior art, and not based on appellant's disclosure). It is important to note that all three elements must be shown to establish a *prima facie* case of obviousness. Thus, if even one element is missing, a *prima facie* case of obviousness does not exist.

The first step in establishing a *prima facie* case of obviousness is presenting evidence that Maziere and Park, in view of Pastey, teach or suggest all of the claim limitations of appellants' present claims. Because none of the three references discloses a method of using HMG-CoA reductase inhibitors to inhibit infection of a cell by a virus in subject *in vivo*, they do not teach or suggest all of the claim limitations found in claim 1. Additionally, the examiner admits that the

cited prior art "does not expressly disclose the employment of HMG-CoA reductase inhibitors in a method of inhibiting infection of a cell by a virus such as RSV in ***a human, a non-human mammal, or a livestock animal.***" And as discussed above, the examiner's argument that the claims read on *in vitro* methods simply does not hold water. Therefore, Maziere, Park, and Pastey fail to establish a necessary element required for a *prima facie* case of obviousness.

Another element of a *prima facie* case of obviousness requires that there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. The examiner states that Pastey teaches "that RhoA-derived peptide inhibits RSV." Appellants believe the examiner is referring to Pastey, Gower *et al.* (C33, PTO-1129 submitted June 11, 2002) rather than Pastey, Crowe *et al.* (C32, PTO-1129 submitted June 11, 2002; Exhhibit H). Regardless, Pastey, Gower *et al.* and Park are directed at solving two completely different problems. The purpose of Park's paper was to examine the effect of HMG-CoA reductase inhibitors on the cholesterol metabolic pathway. On the other hand, Pastey, Gower *et al.* examines the inhibition of RSV syncytium formation using peptides. Thus, these references have very little to do with each other.

Furthermore, in contrast to both Pastey, Gower *et al.* and Park, Maziere discloses an *in vitro* method of using HMG-CoA reductase inhibitors to inhibit ***HIV-1*** expression in a H9 cell line. As mentioned before, Park addresses the use of HMG-CoA reductase inhibitors for the upregulation of ***TGFβ***. Park does not teach that HMG-CoA reductase inhibition of RhoA geranylgeranylation inhibits viral expression. Therefore, Park has no relevance to the inhibition of RSV or HIV-1 expression. Moreover, Pastey, Gower *et al.* is strictly directed toward inhibition of RSV and parainfluenza virus type 3 (PIV-3) using peptides, not HMG-CoA

reductase. The RhoA-derived peptide inhibition is based purely on a process of inhibiting virus attachment of cell-to-cell fusion, while the mechanism for lovastatin action was unstated or assumed to be related to membrane function. Furthermore, neither Park nor Pastey, Gower *et al.* make any mention of the HIV-1 virus. Thus, the examiner has attempted to make a circuitous connection between Maziere and Pastey, Gower *et al.* through Park. However, the approach fails since each reference deals with distinct issues that only peripherally bear up on the claimed invention. Based on the divergent teachings of these three references, a person of ordinary skill in the art would have no motivation to combine Maziere, Park and Pastey, Gower *et al.*

Additionally, the examiner argues that by combining the teachings of Maziere and Park in view of Pastey, Gower *et al.* one with ordinary skill in the art would be motivated to use a nucleoside analog such as AZT in combination with a HMG-CoA reductase inhibitor to inhibit infection of a virus such as RSV. The only reference that mentions a nucleoside analog is Maziere, which states "[m]ost of the drugs used ... such as AZT, are inhibitors of viral replication. However such compounds [appear] to be ***poorly effective*** ...." Emphasis added. One of ordinary skill in the art would understand Maziere not as teaching that a nucleoside analog should be used in combination with HMG-CoA reductase inhibitors, but rather that HMG-CoA reductase inhibitors should be used ***instead*** of nucleoside analogs. Neither Park nor Pastey, Gower *et al.* make any mention of nucleoside analogs. Thus, no motivation remotely exists for one skilled in the art to combine the teachings in the three references to combine a nucleoside analog and HMG-CoA reductase inhibitors.

In response to this line of argument, the examiner submits that appellants are attacking the references individually, which is not proper. This is false. Appellants have shown why ***the individual teachings of the references preclude their logical combination***, in light of the

understanding of those of skill in the art. One ***must*** examine the teachings of the references individually to understand why they are not readily combined as argued by the examiner. Appellants again submit that the examiner is stringing the references together in a tenuous fashion using appellants' own claims as a "road map" to the invention. This is a classic example of "hindsight reconstruction" which is not permitted under the law. *W.L. Gore Assoc., Inc. v. Garlock, Inc.*, 220 USPQ 303, 312-313 (Fed. Cir. 1983) ("To imbue one of ordinary skill in the art with knowledge of the invention in suit, where no prior art reference or references of record convey or suggest that knowledge, is to fall victim to the insidious effect of a hindsight syndrome wherein that which only the inventor taught is used against its teacher.")

The third element required for a *prima facie* showing of obviousness is that in combining references, there be a reasonable expectation of success. Pastey, Crowe *et al.* discloses the discovery that RSV F that interacts with RhoA and facilitates RSV syncytium formation. Pastey, Gower *et al.* discloses a ***peptide*** that spans the F binding domain of RhoA and inhibits RSV syncytium formation. However, Pastey, Gower *et al.* makes no mention of HMG-CoA reductase inhibitors. On the other hand, Park teaches that HMG-CoA reductase inhibitors are capable of inhibiting the ***geranylgeranylation*** of RhoA for the purpose of upregulating transforming growth factor-β (TGFβ), but fails to address peptides. The two references involve two completely distinct mechanisms. Most importantly, ***Park does not present any evidence that inhibition of RhoA geranylgeranylation also inhibits viral expression***. In fact, no mention is made at all whether inhibition of RhoA geranylgeranylation could be successful in inhibiting RSV expression, ***much less inhibition of HIV-1!*** Thus, no person skilled in the art would have a reasonable expectation of success by combining the teachings of Park and Pastey, Crowe *et al.*, in the context of Maziere's teachings.

The examiner has failed entirely to even address this aspect of appellants' previous response. A proper obviousness analysis requires that ***each*** element of a *prima facie* case be established, ***including likelihood of success***. Not only has the examiner advanced a reasonable argument in support of this factor, appellants now have provided clear reasons to believe that there is ***no*** basis for likelihood of success. In light of the final Office Action's silence on this point, appellants submit that the rejection is, and continues to be, improperly maintained. Thus, because the examiner has failed to present a *prima facie* case of obviousness on all three elements, appellants respectfully request reversal of the rejection.

E. Rejection under 35 U.S.C. § 103(a) – Maziere & Park in view of Pastey

Claim 14 stands rejected under §103(a) as being unpatentable over Maziere and Park in view of Pastey, Gower *et al.*. The examiner argues that Maziere and Park in view of Pastey, Gower *et al.* teach the combination of a HMG-CoA reductase inhibitor and an inhibitor of isoprenylation in inhibiting infection of a cell by a virus such as RSV. Appellants respectfully traverse.

Claim 14 is not obvious over Maziere and Park in view of Pastey, Gower *et al.* for the reasons mentioned in section D, above. Moreover, appellants submit the following arguments against the examiner's contention of obviousness.

A *prima facie* case of obviousness requires that Maziere and Park in view of Pastey, Gower *et al.* teach or suggest all of the claim limitations in claim 14. The examiner admits that the employment of HMG-CoA reductase inhibitors in combination with an inhibitor of isoprenylation that is distinct from said HMG-CoA reductase inhibitors in a method of inhibiting infection in a cell by a virus such as RSV is not expressly disclosed in the references. Claim 14

states "[t]he method of claim 1 ... administering to said ***subject*** an inhibitor of isoprenylation that is distinct from said HMG-CoA reductase inhibitor." As emphasized before, a subject is not a cell nor an *in vitro* method. Park only teaches the use of HMG-CoA reductase inhibitors and two inhibitors of isoprenylation ***in vitro***, and Maziere and Pastey are limited to *in vitro* applications as well. Clearly, all the claim limitations in claim 14 have not been suggested or taught by the references. Thus, the examiner has not established an element required for a *prima facie* case of obviousness.

A second element needed to establish a *prima facie* case of obviousness is that there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify or combine the teachings of Maziere and Park in view of Pastey, Gower *et al.* Appellants disagree that any motivation exists to combine the teachings of Maziere and Park in view of Pastey, Gower *et al.* As discussed above, Park teaches the use of isoprenylation inhibitors for the purpose of studying the regulation of TGFβ signaling. Park never discusses inhibition of the RSV virus, nor does it teach that inhibitors of isoprenylation can be used to inhibit infection by any virus much less the RSV virus, much less any mention of HIV-1. Similarly, Maziere does not teach the use of inhibitors of isoprenylation to inhibit the RSV virus, thus potentially implicating Pastey, Gower *et al.* To the contrary, Maziere teaches the use of HMG-CoA reductase inhibitors to inhibit ***HIV-1***, and even then, to inhibit replication, not virus entry through a receptor. Even taking into account Pastey, Gower *et al.*, no evidence is presented in the prior art suggesting that HMG-CoA reductase inhibitors, with or without inhibitors of isoprenylation, should be used to inhibit infection by even the RSV virus. Based on the lack of evidence and the divergent teachings of Park and Maziere, a skilled artisan with knowledge generally available to one skilled in the art

would not be motivated to combine these references. Therefore, a second requirement for a *prima facie* case of obviousness has not been met.

The examiner argues that legal precedent (*Kerkhoven*) supports the obviousness of "combin[ing] two compositions each of which is taught by the prior art to be useful for the same purpose in order to form a third composition that is to be used for the very same purpose." Unfortunately, the flaw with this argument is that HMG-CoA reductase inhibitors ***have not been shown to be useful for treating HIV infections in vivo.*** Thus, the rejection effectively collapses into the same issue discussed above, and appellants again submit that there is no basis for finding obviousness here.

The final element in establishing a *prima facie* case of obviousness requires that there be a reasonable expectation that modifying the teachings of Maziere and Park in view of Pastey, Gower *et al.* would be successful. As mentioned above, Pastey, Gower *et al.* and Park disclose two completely different inhibition mechanisms for two completely different purposes – peptide inhibition of RSV, and HMG-CoA reductase inhibitors. Pastey, Gower *et al.* teaches the use of a peptide to bind to the F protein binding site of RhoA in inhibiting RSV syncytium formation. In contrast, Park teaches the inhibition of geranylgeranylation of RhoA in the upregulation of TGFβ signaling by use of inhibitors of isoprenylation. Inhibition of RSV syncytium formation ***through a RhoA derived peptide*** teaches nothing with regard to possible inhibition of RSV viral infection any other RhoA-related mechanisms. Park does not even mention that inhibition of geranylgeranylation of RhoA by HMG-CoA and isoprenylation inhibitors could inhibit any viral infection, much less RSV, and much, much less HIV-1. Moreover, Maziere does not teach geranylgeranylation in discussing the mechanism in which HMG-CoA reductase inhibitors prevents HIV-1 expression. Simply stated, no evidence exists that shows that inhibition of

geranylgeranylation of RhoA by HMG-CoA and isoprenylation inhibitors reduces RSV or any viral expression. The examiner has produced no evidence that one skilled in the art would be reasonably successful in combining the divergent teachings of Maziere, Park, and Pastey, Gower *et al.* Thus, the prior art cannot be read as providing any reasonable likelihood of success. Accordingly, the examiner has failed to establish the final element necessary for a *prima facie* case of obviousness.

The examiner argues, in response, that there is no showing of unexpected results for the claimed combination. However, unexpected results are only required ***once the examiner has established a proper prima facie case of obviousness***. As should be evident, there is ***no such prima facie case***. Thus, it remains the examiner's burden to explain why *in vitro* studies in HIV-1, when combined with other *in vitro* studies using an entirely different composition result in a ***reasonable*** likelihood of success for treating a human infectious disease. Moreover, the unexpected benefits of the ***combination*** in claim 14 is irrelevant where there is an unexpected benefit for the claim from which claim 14 depends.

Based on the examiner's failure to establish all three of the required elements for a *prima facie* case of obviousness, the Appellants respectfully request that rejection of claim 14 be reversed.

F. Rejection under 35 U.S.C. § 103(a) - Maziere and Park in view of Pastey & Fisher *et al.*

Claim 17 stands rejected under §103(a) as being unpatentable over Maziere and Park in view of Pastey, Gower *et al.* and Fisher *et al.* (Fisher). The examiner argues that Maziere and Park in view of Pastey, Gower *et al.* and Fisher teaches the combination of a HMG-CoA

reductase inhibitor and an antibody composition in inhibiting infection of a cell by a virus such as RSV. Appellants respectfully traverse.

As mentioned above, in order to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success; and (3) the prior art reference (or references when combined) must teach or suggest all the claim limitations. If any one element is missing, a *prima facie* case of obviousness does not exist.

The first element necessary to establish a *prima facie* case of obviousness requires that Maziere and Park in view of Pastey, Gower *et al.* and Fisher teach or suggest all of the claim limitations in claim 17. The examiner admits that none of the references expressly disclose combining HMG-CoA reductase inhibitors with monoclonal or polyclonal antibodies. Fisher is the only reference that contains any discussion about antibodies and only discusses the use of antibodies alone in treating RSV. Fisher does not even discuss the possibility of combining antibodies with other therapies. Additionally, none of the four references teaches the use of HMG-CoA reductase inhibitor in combination with antibody therapies ***in vivo***. Although Fisher teaches administration of antibodies alone *in vivo*, Maziere, Park, and Pastey, Gower *et al.* only discuss administration of HMG-CoA reductase inhibitors *in vitro*. Because Fisher along with Maziere, Park and Pastey, Gower *et al.* fail to suggest all the claim limitations, a *prima facie* case of obviousness has not been established.

Another element required that is required in order for a *prima facie* case of obviousness to exist is that there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify or combine the teachings of Maziere and Park in view of Pastey, Gower *et al.* and Fisher. "The mere fact that references can be combined or modified does not render the resultant combination obvious ***unless the prior art also suggests the desirability of the combination.***" *Manuel of Patent Examining Procedure* (MPEP) § 2143.01 (8th Ed. Rev.). Emphasis added. None of these references make any suggestion of combining antibodies and HMG-CoA reductase inhibitors to inhibit infection by a virus such as RSV. As noted above, the only reference which mentions antibodies is Fisher. which does not teach the use of any other treatment for RSV, only the use of antibodies. Fisher also never suggests the use of antibodies in combination with HMG-CoA reductase inhibitors.

Therefore, the issue is whether one of ordinary skill in the art with knowledge that is generally available would be motivated to combine Maziere and Park in view of Pastey, Gower *et al.* and Fisher. Appellants disagree that one of ordinary skill in the art would be motivated to combine these references. Many therapies have been investigated in treating viral infections, but Fisher does not even mention the use of antibodies in conjunction with any of these therapies let alone use with HMG-CoA reductase inhibitors. There are hundreds of potential therapy combinations for inhibiting viral infections such that a skilled artisan could not possibly know that antibodies could be combined with other therapies if the prior art does not recommend any therapies to use or even suggest that the possibility of such a combination exists. Consequently, Maziere and Park in view of Pastey, Gower *et al.* and Fisher fail to establish an element necessary for a *prima facie* case of obviousness.

The examiner argues that legal precedent (*Kerkhoven*) supports the obviousness of "combin[ing] two compositions each of which is taught by the prior art to be useful for the same purpose in order to form a third composition that is to be used for the very same purpose." Unfortunately, the flaw with this argument is that HMG-CoA reductase inhibitors ***have not been shown to be useful for treating HIV infections in vivo.*** Thus, the rejection effectively collapses into the same issue discussed above, and appellants again submit that there is no basis for finding obviousness here.

The final element in establishing a *prima facie* case of obviousness requires that there be a reasonable expectation that modifying the teachings of Maziere and Park in view of Pastey, Gower *et al.* and Fisher would be successful. Appellants submit that there would be no reasonable expectation of success in combining HMG-CoA reductase inhibitors and antibody compositions in inhibiting infection of a cell by a virus such as RSV. The examiner again presents no evidence that combining HMG-CoA inhibitors and antibodies would reasonably result in success. The result of combining drug therapies is impossible to predict. Much experimentation must occur before a combination of viral therapies proves to be successful. The examiner appears to assume that combining HMG-CoA reductase inhibitors and antibodies will automatically result in successful inhibition of RSV expression. There simply is no basis for such an assumption. Furthermore, none of the four references provide any guidance or recommendations as to whether combining HMG-CoA reductase inhibitors and antibodies would be successful in inhibiting RSV infection. At best, the prior art presents an "obvious to try" situation. Specifically, the combination of HMG-CoA reductase inhibitors and an antibody composition may or may not be successful. However, the PTO's reviewing court has consistently held that "'obvious to try' is not the standard" and "does not render a claim

obvious." *Ecolochem, Inc. v. Southern California Edison Co.*, 227 F.3d 1361, 56 U.S.P.Q.2d 1065 (Fed. Cir. 2000), *In re Roemer*, 258 F.3d 1303, 59 U.S.P.Q.2d 1537 (Fed. Cir. 2001). The Appellants stress that a reading of the prior art cannot in any way provide a ***reasonable*** likelihood of success. As such, Maziere and Park in view of Pastey, Gower *et al.* and Fisher do not establish a reasonable expectation of success as required for a *prima facie* case of obviousness.

The examiner argues, in response, that there is no showing of unexpected results for the claimed combination. However, unexpected results are only required ***once the examiner has established a proper prima facie case of obviousness***. As should be evident, there is ***no such prima facie case***. Thus, it remains the examiner's burden to explain why *in vitro* studies in HIV-1, when combined with other *in vitro* studies using an entirely different composition result in a ***reasonable*** likelihood of success for treating a human infectious disease. Moreover, the unexpected benefits of the ***combination*** in claim 17 is irrelevant where there is an unexpected benefit for the claim from which claim 17 depends.

The examiner has not provided evidence to establish that claim 17 was *prima facie* obvious at the time of filing. Accordingly, appellants respectfully request that the rejection of claim 17 be reversed.

G. Rejection under 35 U.S.C. § 103(a) - Maziere & Park in view of Pastey & Gruber *et al.*

Claim 18 stands rejected under §103(a) as being unpatentable over Maziere and Park in view of Pastey, Gower *et al.* and Gruber *et al.* (Gruber). The examiner argues that Maziere and Park in view of Pastey, Gower *et al.* and Gruber teaches the combination of a HMG-CoA

reductase inhibitor and ribavarin in inhibiting infection of a cell by a virus such as RSV. Appellants respectfully traverse.

In light of the reasons presented in Sections D, E and F, above, Appellants disagree that claim 18 is obvious over Maziere and Park in view of Pastey, Gower *et al.* and Gruber. Furthermore, Appellants submit the following arguments against the examiner's assertion of obviousness.

One element necessary to establish a *prima facie* case of obviousness requires that Maziere and Park in view of Pastey, Gower *et al.* and Gruber teach or suggest all of the claim limitations in claim 18. The examiner admits that none of the references expressly disclose combining HMG-CoA reductase inhibitors with ribavarin. Gruber is the only reference that contains any discussion about ribavarin and only discusses the use of ribavarin with human immunoglobin (IVIG) in treating RSV. Furthermore, none of the four references teaches the use of HMG-CoA reductase inhibitor in combination with ribavarin ***in vivo***. Although Gruber teaches administration of ribavirin *in vivo*, Maziere, Park, and Pastey, Gower *et al.* only discuss administration of HMG-CoA reductase inhibitors *in vitro*. Because Gruber along with Maziere, Park and Pastey, Gower *et al.* fail to suggest all the claim limitations, a *prima facie* case of obviousness has not been established.

Another element required that is required in order for a *prima facie* case of obviousness to exist is that there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify or combine the teachings of Maziere and Park in view of Pastey, Gower *et al.* and Gruber. None of these

references make any suggestion of combining ribavarin and HMG-CoA reductase inhibitors to inhibit infection by a virus such as RSV.

Therefore, the relevant inquiry is whether knowledge that is generally available to one of ordinary skill in the art would supply the motivation to combine Maziere and Park in view of Pastey, Gower *et al.* and Gruber. Appellants submits that one skilled in the art would not be motivated to combine Maziere and Park in view of Pastey, Gower *et al.* and Gruber. Gruber teaches the use of ribavirin in combination with IVIG or "another agent". However, Appellants reiterate that many therapies and agents are available in treating viral infections. Gruber provides no examples of "another agent" nor does it even mention HMG-CoA reductase inhibitor as such an agent. Although Gruber mentions that ribavirin may be used in combination with "another agent", the fact that it makes no suggestion of any particular combination of ribavirin and "another agent" besides IVIG does not provide any information to skilled artisans that would motivate them to specifically combine HMG-CoA reductase inhibitor with ribavirin. Additionally, one skilled in the art would not be motivated to specifically combine HMG-CoA reductase inhibitor with ribavirin to inhibit RSV when the divergent teachings of Maziere and Park in view of Pastey, Gower *et al.* do not disclose the use of HMG-CoA reductase inhibitor to inhibit infection by the RSV virus. As such, Maziere and Park in view of Pastey, Gower *et al.* and Gruber fail to establish an element required for a *prima facie* case of obviousness.

The examiner argues that legal precedent (*Kerkhoven*) supports the obviousness of "combin[ing] two compositions each of which is taught by the prior art to be useful for the same purpose in order to form a third composition that is to be used for the very same purpose." Unfortunately, the flaw with this argument is that HMG-CoA reductase inhibitors ***have not been shown to be useful for treating HIV infections in vivo.*** Thus, the rejection effectively collapses

into the same issue discussed above, and appellants again submit that there is no basis for finding obviousness here.

A third element in establishing a *prima facie* case of obviousness requires that there be a reasonable expectation that modifying the teachings of Maziere and Park in view of Pastey, Gower *et al.* and Gruber would be successful. One skilled in the art would have no reasonable expectation of success by modifying the teachings of Maziere and Park in view of Pastey, Gower *et al.* and Gruber. Gruber states a "[c]ombination of ribavirin with another agent ***might*** provide improved protection against RSV infection." Emphasis added. Although Gruber describes a successful combination of ribavarin and IVIG, it provides no evidence that a combination of ribavirin and "another agent" would provide the same results. Additionally, Gruber provides no guidance as to which combination of ribavirin and another agent besides IVIG would reasonably expected to be successful. The language, "might", in no way would assure a skilled artisan that combining ribavirin with any agent, let alone a HMG-CoA reductase inhibitor, would reasonably result in successful inhibition by RSV.

Additionally, the examiner argues that additive therapeutic effects would be reasonably expected. This simply is not the case. Combining viral therapies is a highly unpredictable art. Trial and error is often required to determine the proper combination of therapies. Evidence of such unpredictability is observed in Gruber. Gruber mentions that ribavirin and IVIG were tested *in vitro* to look for additive effects, but found that additive effects were ***minimal*** in inhibiting RSV. Gruber appears to show that the success in combining ribavirin and IVIG *in vivo* was ***unexpected*** in view of these *in vitro* results. Furthermore, it appears that some other *in vivo* interaction ***specific*** to IVIG and ribavirin is responsible for the additive effect as shown by the statement "[f]urther studies are indicated to determine how IVIG and ribavirin interact with

local antibody and cellular elements to limit RSV infection." Clearly, the success in Gruber was not due to expected additive results, but as a result of unpredictable trial and error. Gruber gives no indication that similar success would come about from the combination of HMG-CoA indicators and ribavarin. As mentioned in section F above, the prior art, at most, describes an "obvious to try" situation which does not render a claim obvious. Accordingly, the examiner has not established an element necessary for a *prima facie* case of obviousness.

The examiner argues, in response, that there is no showing of unexpected results for the claimed combination. However, unexpected results are only required ***once the examiner has established a proper prima facie case of obviousness***. As should be evident, there is ***no such prima facie case***. Thus, it remains the examiner's burden to explain why *in vitro* studies in HIV-1, when combined with other *in vitro* studies using an entirely different composition result in a ***reasonable*** likelihood of success for treating a human infectious disease. Moreover, the unexpected benefits of the ***combination*** in claim 18 is irrelevant where there is an unexpected benefit for the claim from which claim 18 depends.

In view of the examiner's failure to satisfy any of the three elements needed for a *prima facie* case of obviousness, the Appellants respectfully request that the rejection to claim 18 be reversed.

H. Claims 3-6 are Separately Patentable over the Art of Record

Claims 3-6 are rejected over Maziere, Pastey, Gower *et al.* and Park. The teachings of those references are discussed extensively, above. However, regardless of what those references teach, they cannot address the limitations of claims 3-6. Claim 3 recites that the subject is or will become immunusuppressed, while claim 4 recites that the subject being treated suffers from

severe combined immunodeficiency. These aspects of the invention are nowhere mentioned or alluded to in the references, particularly because they do not address treatment of individuals with immunosuppressing diseases. Thus, the failure of these embodiments to be stated or suggested by the cited art renders rejection of these claims improper.

Similarly, claim 5 recites that a subject is or will be taking immunosuppressive drugs and is or will be a transplant recipient. Again, the references are silent on the inclusion of immunosuppressive drugs in any treatment regimen, much less that the recipient of such drugs will be the subject of a transplant. Therefore, given the complete absence of disclosure from the references of these features, it is a legal impossibility that the present claims can be rendered obvious thereby.

As such, appellants submit that for these additional reasons, the invention as claimed in claims 3-6 cannot is not obvious. Reversal on these grounds also is requested.

X. Conclusion

It is respectfully submitted, in light of the above, that all of the pending claims are in condition for allowance. Appellant, therefore, requests that the Board reverse each of the pending grounds for rejection.

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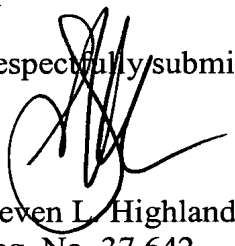
Respectfully submitted,



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Date: May 24, 2004

APPENDIX 1: PENDING CLAIMS

1. A method of inhibiting infection of a cell by a virus in a subject comprising the step of administering to said subject an inhibitor of HMG-CoA reductase.

2. The method of claim 1, wherein said subject does not have an existing viral infection.

3. The method of claim 2, wherein said subject is or will become immunosuppressed.

4. The method of claim 3, wherein said subject suffers from severe combined immundeficiency.

5. The method of claim 3, wherein said subject is taking or will take immunosuppressive drugs.

6. The method of claim 5, wherein said subject is or will be a transplant recipient.

7. The method of claim 1, wherein said subject has an existing viral infection.

8. The method of claim 1, wherein said virus is selected from the group consisting of human immunodeficiency virus, human respiratory syncytial virus (RSV), parainfluenza virus, ebola virus, measles, canine distemper, Newcastle's Disease virus, bovine RSV, ovine RSV and turkey rhinotracheitis.

9. The method of claim 1, wherein said subject is a human.

11. The method of claim 9, wherein said virus is respiratory syncytial virus.

13. The method of claim 1, wherein said inhibitor is selected from the group consisting of lovastatin, simvastatin, fluvastatin, atorvastatin, pravastatin and mevastatin.

14. The method of claim 1, further comprising administering to said subject an inhibitor of isoprenylation that is distinct from said HMG-CoA reductase inhibitor.

15. The method of claim 10, further comprising administering to said subject a nucleoside analog composition.

16. The method of claim 15, further comprising administering to said subject a protease inhibitor.

17. The method of claim 11, further comprising administering to said subject an antibody composition that binding immunologically to RSV.

18. The method of claim 11, further comprising administering to said subject ribavarin.

19. The method of claim 1, wherein said subject is a non-human mammal.

20. The method of claim 19, wherein said non-human mammal is a livestock animal.

Biomed & Pharmacother (1994) 48, 63-67

Lovastatin inhibits HIV-1 expression in H9 human T lymphocytes cultured in cholesterol-poor medium

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Summary – The effects of the HMG-Coenzyme A reductase inhibitor lovastatin on HIV-1 expression and sterol synthesis have been investigated in the human H9 lymphocytic cell line. To this purpose, sterol synthesis from ^{14}C-acetate, cell multiplication and reverse transcriptase activity have been measured in parallel at various times after cell infection by HIV-1. It was found that nine days after viral loading, lovastatin inhibited both sterol synthesis and viral multiplication as assessed by the reverse transcriptase activity. Since HIV infection has been shown to induce alterations in membrane cholesterol content, suggesting that the virus cycle may be partially dependent upon cellular cholesterol, inhibitors of cholesterol synthesis could be an interesting way of research in order to slower HIV propagation.

HIV-1 / lovastatin / cholesterol / reverse transcriptase

Introduction

Several strategies are currently being developed in order to inhibit HIV propagation in humans, some of them based upon pharmacotherapy using antiviral agents. Most of the drugs used to this purpose, such as AZT, are inhibitors of viral replication [1, 2]. However, such compounds appeared to be poorly effective in the treatment of AIDS [3, 4]. A critical review of the literature concerned with lipid metabolism in cells infected with HIV indicated that in several experimental models, significant lipid alterations are observed as compared to uninfected cells. Indeed, the membrane cholesterol/phospholipid ratio has been reported to be markedly increased in peripheral blood mononuclear cells infected with HIV [5]. It is also interesting to note that the cholesterol/phospholipid ratio of the virus envelope is much higher than that of cellular membranes [6]. Moreover, plasma cholesterol and low density lipoprotein (LDL) levels are low in AIDS patients [7, 8], suggesting that HIV could use exogenous as well as endogenous sources of cholesterol to achieve enrichment of its envelope in cholesterol. Several works have been previously concerned with the possibility of slowing HIV propagation by reducing cholesterol in cellular membranes. Among them, the use of phospholipid emulsions able *in vitro* to induce cholesterol efflux from cells, such as AL 721, has been explored [9].

Lovastatin belongs to the class of the hydroxy methylglytaryl Coenzyme A (HMG-CoA) reductase inhibitors initially described by Endo *et al* [10]. Such compounds strongly reduce cellular cholesterol synthesis by competition with the natural substrate of the enzyme [11]. It thus appeared of interest to test whether such inhibitors of cholesterol synthesis could be of use in order to reduce the replication rate of HIV in infected cells, based on the working hypothesis that cholesterol is an important requirement for building infectious forms of the virus. We thus investigated the effect of lovastatin on cholesterol synthesis and reverse transcriptase (RT) activity in the human lymphocytic cell line H9 [12] infected with HIV-1. It was found that lovastatin, at a concentration close to that utilized in the therapy of atherosclerosis in humans, was significantly effective in reducing HIV replication at early stages of infection.

Materials and methods

Chemicals

Lovastatin was a generous gift from Merck Laboratories. RPMI 1640 medium and antibiotics were from Serva (Heidelberg, Germany). ^{14}C-acetate (55 mCi/mol) was purchased from the CEA (Saclay, France). [methyl-^{3}H] thymidine 5′ triphosphate (^{3}H-TTP, 30Ci/mol) was obtained from Amersham France (Les Ulis, France). All other chemicals were obtained from Sigma (St Louis, MO, USA) and were of the purest available grade.

Cell culture

H9 human T lymphocytes derived from the HUT 78 cell line [12] are maintained in RPMI 1640 medium supplemented with 10% pooled human serum AB (SAB). The cells, seeded at a density of $1.5 \pm 0.25 \times 10^5/cm^2$ in vented upright Costar 3056 flasks, are regularly grown at 37°C in a humidified atmosphere of 5% CO_2. For experiments dealing with the effect of lovastatin, H9 cells were first sub-cultured in RPMI 1640 medium supplemented with only 1% SAB, in order to reduce exogenous cholesterol supply. After five subcultures under this reduced supply, the cellular growth rate appeared to be reproducible enough for experiments.

H9 cell-loading with HIV

The H9 cells were washed three times then resuspended in 1 ml RPMI 1640 culture medium devoid of serum, in the presence of HIV-1 particles at a concentration corresponding to about 10^5 cpm/ml reverse transcriptase activity, determined as further described. The viral particles were collected from an H9 cell line producing HIV-1 (cell line HTLV IIIB) [12]. The cells were incubated with the viral suspension for 60 min at room temperature to allow infection, washed three times, then resuspended in 5 ml RPMI medium supplemented with 1% SAB. These HIV-1 infected cells were seeded at a density of 0.5×10^6/ml in 24-well Costar plates.

Cell treatment with lovastatin

Lovastatin (3×10^{-7} M final concentration) was added to the culture medium as concentrated ethanolic solution (final concentration of ethanol: 0.1%) at day 1 after cell loading with HIV. The medium was replaced each day by new medium containing the same lovastatin concentration. At days 5 and 9 after cell infection, sterol synthesis and protein determination were studied as described below, while manual cell counting (means of fours determinations) was carried out on an aliquot of the cell suspension. The reverse transcriptase activity was studied on the culture medium as described elsewhere.

Sterol synthesis

Sterol synthesis was studied as previously described [13], using 0.04 µCi/ml sodium [^{14}C]acetate. Cells were incubated for 4 h at 37°C with the precursor, then washed three times with a phosphate-buffered solution pH 7.4, and harvested with rubber policemen. Lipid analysis was achieved by thin layer chromatography using hexane/diethylether/acetic acid (70:30:2, vol/vol) as solvent. After autoradiography, the radioactive spots were cut and counted by liquid scintillation using a Beckman LS 6000 L counter. Control experiments with ethanol alone were also performed. Results, calculated as pmol of precursor incorporated per mg of cell protein, are expressed as % of controls. Protein determination was carried out according to Lowry *et al* [14].

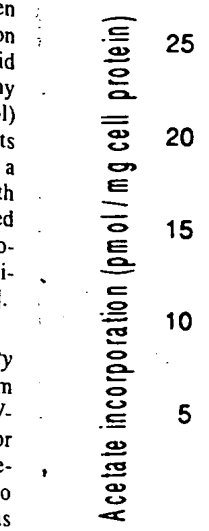
Assessment of reverse transcriptase (RT) activity

The RT activity was measured on the culture medium as previously described [15]. At days 5 and 9 after HIV-1 infection, the culture medium was centrifuged for 10 min at 1 300 × g. The clarified supernatant was recovered and centrifuged again for 2 h at 40 000 × g to pellet virus particles. The subsequent virus pellet was resuspended on ice in 0.02 ml 0.05 M Tris-HCl, 0.3 M KCl, 0.0014 M dithiothreitol, pH 7.5, 0.015 mg/ml polyadenylic acid, 0.15 mg/ml [12-18] oligothymidylic acid, and 3 µCi ^{3}H-TTP. Samples were incubated for 1 h at 37°C, and the reaction was stopped on ice by addition of 1 ml of 5% trichloracetic acid (TCA)/ 0.1 mM sodium pyrophosphate and 0.25 ml of a 0.5 mg/ml yeast tRNA solution. After further addition of 3.5 ml of a 20% TCA solution, samples were kept at 4°C for 15 min then spotted onto a Whatman no 3 disk. The radioactivity incorporated in TCA-precipitable material was measured by liquid scintillation, and expressed as cpm/ml of culture medium.

Results and Discussion

The determination of sterol synthesis, cell number and RT activity were initially performed at days 5, 9 and 12 after H9 T cell infection by HIV-1. However, in our experimental conditions, the number of cells was dramatically reduced at day 12, which did not allow reproducible results. Therefore, in the present work, we only present the data obtained at 5 and 9 days after infection.

Figure 1 displays the effect of 3×10^{-7} M lovastatin on sterol synthesis by HIV-1 infected H9 human T lymphocytes. In cells cultured in the presence of lovastatine, ^{14}C-acetate incorporation into sterols was reduced by about 70 and 65% at days 5 and 9, respectively. Interestingly, this inhibitory effect of lovastatin on sterol synthesis is much less marked in our experimental model than that previously reported for HMG-CoA reductase inhibitors in various normal [16, 17] or transformed [18] cell lines. Indeed, the ID_{50} (50% inhibition) of lovastatin usually ranges from 5 ×



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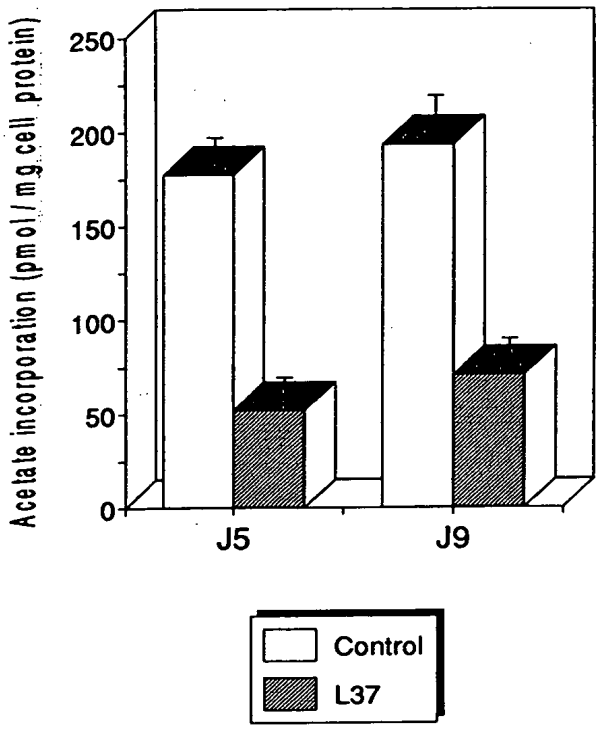

Fig 1. Effect of 3×10^{-7} M lovastatin on sterol synthesis from ^{14}C-sodium acetate by H9 cells at days 5 and 9 after infection by HIV-1. After a 24-h pre-culture in DMEM medium supplemented with 2% Ultroser G, in the absence or in the presence of 3×10^{-7} M lovastatin, cells were washed three times and incubated for 4 h with 0.04 mCi/ml ^{14}C-acetate. After washing, cells were harvested and lipid analysis was performed by thin layer chromatography as described in *Materials and Methods*. Results are expressed as pmol of precursor incorporated/mg cell protein. Means of four experimental values ± sd.

10^{-9} to 5×10^{-8} M [11], and almost total inhibition of sterol synthesis is observed for concentrations of the drug above 10^{-7} M [18]. It must be stressed that sterol synthesis is significantly (about 1.5-fold) increased at the early stage (day 5) of the infection in HIV-1-loaded H9 cells as compared to uninfected cells cultured in 1% SAB. Moreover, uninfected H9 cells are more sensitive to the inhibitory effect of lovastatin on sterol synthesis than HIV-1-infected cells (data not shown). This suggests that viral infection could induce changes in cellular cholesterol metabolism in relation to the high amounts of cholesterol required for the building of the virus envelope.

Figure 2 displays the effect of 3×10^{-7} M lovastatin on the cell number (fig 2a) and on the RT activity (fig 2b) in HIV-1 infected H9 cells. It can be observed that at days 5 and 9, the number of cells/well was significantly higher in cultures treated with lovastatin, suggesting that the cytotoxic action of HIV-1 was less marked than in controls (fig 2a). This is confirmed by data presented in figure 2b, which indicate that the RT activity was about 10-fold reduced in cells treated with lovastatin 3×10^{-7}.

From these results, it can be concluded that lovastatin markedly inhibited HIV-1 expression in H9 human T lymphocytes, as assessed by measurement of the RT activity at days 5 and 9 after infection. Under our experimental conditions (culture medium supplemented with only 1% human serum), it was not possible to follow the expression of the virus on a larger time scale, because of the rapid decrease in cell number in infected cultures after days 11-12, which did not allow to obtain reproducible results. It is therefore difficult to extrapolate to physiological conditions. However, our results provide interesting indications about the role of cholesterol in HIV replication. Indeed, a relatively moderate (about 60%) decrease in endogenous sterol synthesis significantly impaired viral expression in infected lymphocytes cultured in medium containing low amounts of exogenous cholesterol (1% of the blood cholesterol concentration).

The mechanisms by which lovastatin inhibits HIV-1 expression in lymphocytes can probably be related, at least partially, to the inhibition of sterol synthesis. Indeed, the high cholesterol/phospholipid ratio previously reported both in infected cells [5] or in the viral envelope [6] strongly suggests that the HIV infective cycle requires cholesterol. This cholesterol may be obtained either from exogenous sources (mainly LDL), as suggested by the low level of LDL usually observed in blood from patients with AIDS [7, 8], and/or from endogenous cholesterol. As a matter of fact, cellular cholesterol depletion by phospholipid vesicles such as AL 721 has already been envisaged as a therapeutic tool in AIDS [9]. However, the results obtained in therapeutic trials with AL 721 are still controversial [19]. In view of our results, it might be supposed that endogenous cholesterol synthetized by cells (including white blood cells), could be integrated in the virus envelope. This is, to our knowledge, the first time that the role of endogenous cholesterol in HIV expression has been suggested. However, other hypotheses could be raised to explain the inhibitory effect of lovastatin. Among them, one can suppose that the intracellular virus cycle might, at an unknown stage, interfere with cellular me-

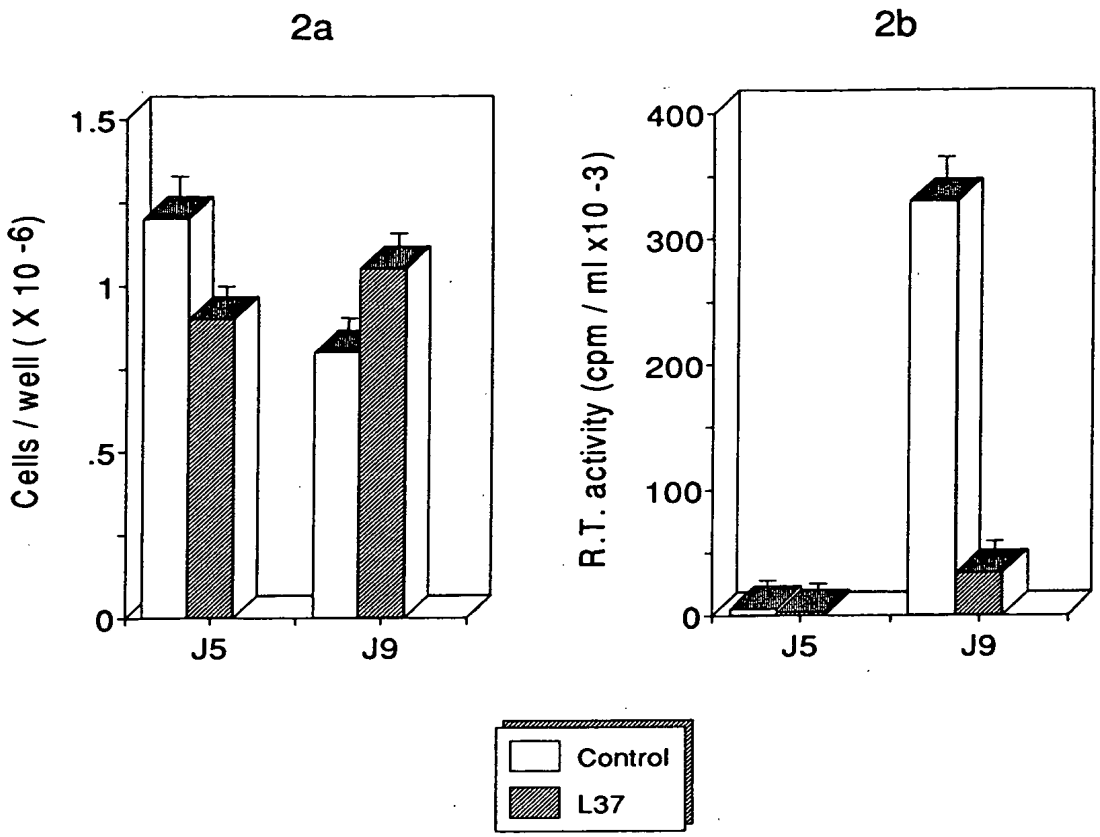

Fig 2. Effect of 3×10^{-7} M lovastatin of cell proliferation (fig 2a) and reverse transcriptase (RT) activity (fig 2b) at days 5 and 9 after infection of H9 cells by HIV-1. Cell proliferation was determined by manual counting (means of four determinations) and expressed as cells $\times 10^{-6}$/culture well. The RT activity was studied in the culture medium as described in *Materials and Methods* and expressed as cpm $\times 10^{-3}$/ml of medium. Means of three experimental values ± sd.

tabolism(s) requiring isoprenoids, which are also important products derived from mevalonate [20], the synthesis of which is impaired by lovastatin.

Conclusion

HMG-CoA reductase inhibitors such as simvastatin (Zocor^{R}) are currently used in the treatment of hypercholesterolemia to slower the progression of atherosclerotic lesions [11]. These drugs are well tolerated and have only slight side effects [21, 22]. It is worth noting that the lovastatin concentration used in our experiments (3×10^{-7} M) is in the upper range of the blood concentrations measured in treated patients [23]. We thus suggest that the use of HMG-CoA reductase inhibitors should be considered as a potential therapeutic method of slowing the HIV infective cycle in AIDS patients. In any case, the striking effect of lovastatin under our experimental conditions raises the possibility of utilizing this type of drug to potentiate more classical therapies.

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3-Hydroxy-3-methylglutaryl CoA reductase inhibitors up-regulate transforming growth factor-β signaling in cultured heart cells via inhibition of geranylgeranylation of RhoA GTPase

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ABSTRACT **Transforming growth factor-β (TGFβ) signaling has been shown to play a role in cardiac development as well as in the pathogenesis of cardiovascular disease. Prior studies have suggested a relationship between cholesterol metabolism and TGFβ signaling. Here we demonstrate that induction of the cholesterol metabolic pathway by growth of embryonic chicken atrial cells in medium supplemented with lipoprotein-depleted serum coordinately decreased the expression of the TGFβ type II receptor (TGFβRII), TGFβ$_1$, and TGFβ signaling as measured by plasminogen activator inhibitor-1 (PAI-1) promoter activity. Inhibition of the cholesterol metabolic pathway by the hydrophobic 3-hydroxy-3-methylglutaryl CoA (HMGCoA) reductase inhibitors, simvastatin and atorvastatin, reversed the effect of lipoprotein-depleted serum and up-regulated TGFβRII expression, whereas the hydrophilic HMGCoA reductase inhibitor, pravastatin, had no effect. Simvastatin stimulated the expression of TGFβRII, TGFβ$_1$, and PAI-1 at the level of transcription. Experiments using specific inhibitors of different branches of the cholesterol metabolic pathway demonstrated that simvastatin exerted its effect on TGFβ signaling by inhibition of the geranylgeranylation pathway. C3 exotoxin, which specifically inactivates geranylgeranylated Rho GTPases, mimicked the effect of simvastatin on PAI-1 promoter activity. Cotransfection of cells with a PAI-1 promoter-reporter and a dominant-negative RhoA mutant increased PAI-1 promoter activity, whereas cotransfection with a dominant-active RhoA mutant decreased PAI-1 promoter activity. These data support the conclusion that TGFβ signaling is regulated by RhoA GTPase and demonstrate a relationship between cholesterol metabolism and TGFβ signaling. Our data suggest that in patients treated with HMGCoA reductase inhibitors, these agents may exert effects independent of cholesterol lowering on TGFβ signaling in the heart.**

Transforming growth factor-β (TGFβ) signaling plays an important role in cardiac development, cardiac hypertrophy, ventricular remodeling, and the early response to myocardial infarction (1–2). Data that correlate severe coronary artery disease with levels of circulating activated TGFβ suggest that TGFβ signaling might also play a role in atherogenesis (3–5). Studies that demonstrate that TGFβ is capable of regulating the expression of low-density lipoprotein (LDL) receptors and the incorporation of ^{14}C-acetate into cholesterol suggest that TGFβ signaling might play a role in regulating cholesterol metabolism (6, 7). Furthermore, in aortas of cholesterol-fed Watanabe rabbits, levels of TGFβ$_1$ are increased (8). These data suggest a relationship between cholesterol metabolism, TGFβ signaling, and cardiovascular disease.

The cholesterol metabolic pathway may be stimulated by depriving cells of lipoproteins and inhibited by 3-hydroxy-3-methylglutaryl CoA (HMGCoA) reductase inhibitors (9). Farnesylpyrophosphate (FPP) represents a branchpoint in the cholesterol metabolic pathway. Not only does it serve as a precursor to cholesterol, but it is also a precursor to ubiquinone, dolichol phosphate, and geranylgeranylpyrophosphate (GGPP), which is required for the posttranslational lipidation and membrane localization of small GTP-binding proteins such as Rho family members. FPP itself is required for the lipidation and membrane localization of Ras (10, 11).

Small GTP-binding proteins play a major role in the regulation of the cell cycle and in the control of gene expression (10, 11). We have recently shown that the posttranslational lipidation of membrane-associated proteins is a regulatable process (12). Thus induction of the cholesterol metabolic pathway increased the extent of lipidation and membrane localization of Ras and the expression of proteins involved in second messenger pathways. Inhibition of the cholesterol metabolic pathway by HMGCoA reductase inhibitors interfered with farnesylation and membrane localization of Ras and reversed the effects of induction of the cholesterol metabolic pathway on gene expression (12–14).

TGFβ signaling is mediated via the interaction of two different TGFβ receptor subtypes, both containing serine/threonine kinase domains (15, 16). On binding of TGFβ to the TGFβ type II receptor (TGFβRII), the receptor is autophosphorylated. In the presence of ligand, TGFβRII forms a complex with the TGFβ type I receptor and catalyzes its phosphorylation. The TGFβRI then interacts with downstream signaling factors such as Smad proteins. The plasminogen activator inhibitor-1 (PAI-1) promoter is one of the major targets of Smad proteins.

The use of HMGCoA reductase inhibitors to study the regulation of cholesterol metabolism and TGFβ signaling has important clinical implications (17). HMGCoA reductase inhibitors are in wide clinical use for the treatment and prevention of coronary artery disease. Recent data have suggested that these drugs might have effects on coronary risk and cellular physiology that are independent of cholesterol lowering (17, 18). Six HMGCoA reductase inhibitors are currently in clinical use (19, 20). They demonstrate markedly different hydrophobicities: simvastatin is the most hydrophobic and pravastatin the most hydrophilic. Several studies have suggested that these differences in hydrophobicity might be related to differences in the ability of the HMGCoA reductase

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Abbreviations: LDL, low-density lipoprotein; HMGCoA, 3-hydroxy-3-methylglutaryl CoA; TGFβ, transforming growth factor-β; TGFβRII, TGFβ type II receptor; LPDS, lipoprotein-depleted serum; PAI-1, plasminogen activator inhibitor-1; FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate.

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ıibitors to mediate nonlipid-lowering effects (19). Several ıdies have suggested that HMGCoA reductase inhibitors er the expression of TGFβ_1 (21, 22). We report here a lationship between cholesterol metabolism and TGFβ sig- ling in cultured embryonic chicken atrial cells. In these cells, hibition of the cholesterol metabolic pathway by HMGCoA ductase inhibitors induces the coordinate up-regulation of e expression of TGFβRII and its ligand, TGFβ_1, and an crease in TGFβ signaling. This effect is independent of olesterol lowering and is caused by interference of the drophobic HMGCoA reductase inhibitors with the post- nslational geranylgeranylation of a member of the Rho nily of small GTP-binding proteins.

MATERIALS AND METHODS

Reagents. Cell culture media and supplies were from Life chnologies (Grand Island, NY). A monoclonal antibody to ıFβRII was from Transduction Laboratories (Lexington, ') and an anti-human c-myc antibody was from PharMingen. ıvastatin, atorvastatin, and simvastatin were gifts from ıstol-Myers Squibb. The squalene synthase inhibitor, TMD, s a kind gift from Thomas Spencer (Dartmouth College, nover, NH) (23); FTI-277, an inhibitor of farnesyltrans- ase (24), and GGTI-298, an inhibitor of geranylgeranyl- nsferase I (25), were kind gifts from Said Sebti (University South Florida, Tampa, FL).

'lasmids. pTGFβRII-500/36-Lux and phTG5 were gifts m Seong-Jin Kim (Laboratory of Chemoprevention, Na- nal Cancer Institute, Bethesda, MD) (26, 27) and p3TP-Lux ıtaining the putative TGFβ responsive region of the human I-1 promoter was a gift from Joan Massague (Memorial an–Kettering Cancer Center, NY) (28). phTGFβ5-Lux was ıerated by subcloning a smaller *Kpn*I-*Bam*HI fragment of I'G5 into *Kpn*I-*Bam*HI sites of pXP2, a promoterless lucif- se vector (12). pGEX2F-C3 was a gift from Larry Feig ıfts University, Boston, MA) (29). pRK5 myc-L63RhoA, DNA3 myc-N19RhoA, and pEFmyc-C3 were gifts from n Hall (University College London). pCMVβgal was from ONTECH.

'reparation of Lipoprotein-Depleted Serum (LPDS). LPDS prepared as described previously (12).

'rimary Culture of Chicken Embryonic Heart Cells. Heart s were cultured from embryos 14 days *in ovo*. Atrial cultures e prepared by a modification of the method of DeHaan) as described previously (14).

urification of C3 Exotoxin. C3 exotoxin was overproduced *scherichia coli* transformed with pGEX2F-C3 and purified iescribed by Dillon and Feig (29).

ımmunoblot Analysis. Equal amounts of protein from ex- ts of cultured heart cells were subjected to SDS/PAGE, ɔwed by electroblotting onto a polyvinylidene difluoride ıbrane (Schleicher & Schuell). Blots were probed with the cated primary antibody followed by a secondary antibody iugated with horseradish peroxidase and visualized by ıiluminescence.

ransfection of Cells. LipofectAMINE Plus (Life Technol- s, Grand Island, NY) was used according to the manufac- r's protocol with slight modifications. A total of 2 μg of mid DNA, including 0.1 μg of pCMVβgal, was used for sfection of cells cultured in 60-mm dishes to 80% conflu- . Cells were incubated with the liposome-DNA complex in ium supplemented with 6% Nu-serum for 5 h. Cells were ıed and allowed to recover for 12 h in medium supple- ted with 6% FCS. Medium was removed and cells incu- d for 16 h, as indicated in the figure legends. Luciferase β-galactosidase assays were carried out as described by ıbel *et al.* (31). Data were presented as the mean value M and analyzed by Student's *t* test where indicated.

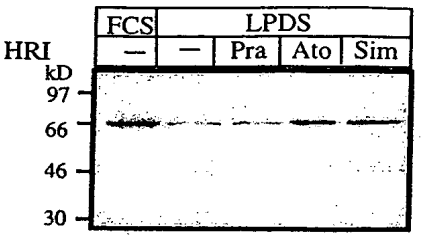

FIG. 1. Regulation of TGFβRII expression by the cholesterol metabolic pathway. Embryonic chicken atrial cells were cultured in media with either FCS or LPDS and treated with various HMGCoA reductase inhibitors each at 10 μM for 16 h. Lane 1, FCS; lane 2, LPDS; lane 3, LPDS plus pravastatin (Pra); lane 4, LPDS plus atorvastatin (Ato); lane 5, LPDS plus simvastatin (Sim). Thirty micrograms of crude cell extract were subjected to SDS/PAGE followed by immunoblotting with a TGFβRII antibody. Data are typical of four similar experiments.

RESULTS

Effect of Regulating the Cholesterol Metabolic Pathway on the Expression of TGFβRII in Cultured Heart Cells. To determine the effect of induction of the cholesterol metabolic pathway on TGFβ signaling, we measured levels of TGFβRII by immunoblot analysis of proteins from embryonic chicken atrial cells grown in media supplemented with either FCS or LPDS (Fig. 1). The level of TGFβRII was markedly decreased in cells grown in LPDS compared with that in cells grown in FCS. To determine whether inhibition of the cholesterol metabolic pathway by HMGCoA reductase inhibitors reversed the effect of LPDS on TGFβRII expression, cells were incubated in LPDS plus 10 μM either pravastatin, atorvastatin, or simvastatin. Both atorvastatin and simvastatin completely reversed the effect of LPDS on the expression of TGFβRII. Pravastatin had no effect on LPDS inhibition of TGFβRII expression (Fig. 1).

Effect of Regulating the Cholesterol Metabolic Pathway on TGFβRII and TGFβ$_1$ Promoter Activity. To determine whether the regulation of TGFβRII expression occurred at the level of transcription, TGFβRII promoter activity was measured in cells transiently transfected with a TGFβRII promoter–luciferase reporter. Growth in LPDS decreased TGFβRII promoter activity by 73 ± 7% (n = 4) compared with cells grown in FCS; simvastatin reversed this effect (Fig. 2).

TGFβ$_1$ promoter activity was measured in cells transfected with a TGFβ$_1$ promoter–luciferase reporter. Growth in medium supplemented with LPDS decreased TGFβ$_1$ promoter

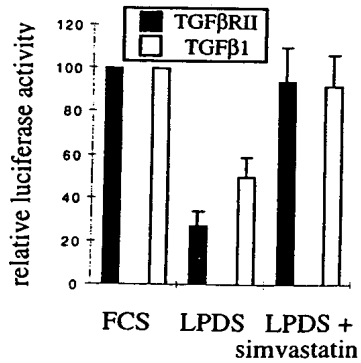

FIG. 2. Coordinate regulation of TGFβRII and TGFβ$_1$ promoter activity by control of the cholesterol metabolic pathway. Cells were transfected with either pTGFβRII-500/36-Lux (■) or phTGFβ5-Lux (□) plus pCMVβgal. After recovery, cells were incubated for 16 h in media with either FCS, LPDS, or LPDS plus simvastatin (20 μM). Luciferase activity was normalized to β-galactosidase activity. Data are plotted as the mean ± SEM of three independent experiments.

activity by 50 ± 9% (n = 3) compared with control. This effect was completely reversed by simvastatin (Fig. 2). Hence factors that regulate the cholesterol metabolic pathway coordinately regulate the expression of both TGFβRII and TGFβ$_1$.

Up-Regulation of TGFβRII and TGFβ$_1$ Expression and TGFβ Signaling by Simvastatin Is Mediated by Inhibition of the Geranylgeranylation Pathway. To identify which of the FPP-dependent pathways played a role in the regulation of TGFβRII and TGFβ$_1$ expression, TMD, an inhibitor of squalene synthase, was used to block cholesterol biosynthesis (23); FTI-277, an inhibitor of farnesyltransferase, to block protein farnesylation (24); and GGTI-298, an inhibitor of geranylgeranyltransferase, to block protein geranylgeranylation (25). Data summarized in Fig. 3*A* demonstrated that neither TMD nor FTI-277 had an effect on TGFβRII and TGFβ$_1$ promoter activities (Fig. 3*A*, lanes 3 and 4), whereas GGTI-298 increased TGFβRII and TGFβ$_1$ promoter activities by 4.5 ± 0.48-fold and 1.9 ± 0.16-fold (n = 3), respectively (Fig. 3*A*, lane 5). Similar results were obtained from immunoblot analysis of the expression of TGFβRII protein (Fig. 3*B*). Thus, the coordinate up-regulation of the expression of TGFβRII and TGFβ$_1$ by simvastatin is mediated by the inhibition of protein geranylgeranylation.

If simvastatin acts by inhibition of the geranylgeranylation pathway, addition of GGPP, the substrate for geranylgeranyltransferase, should reverse the up-regulation of TGFβRII expression induced by simvastatin. Incubation of cells in LPDS with simvastatin plus GGPP reversed the effect of simvastatin on TGFβRII promoter activity (Fig. 4*A*) and on TGFβRII protein (Fig. 4*B*), whereas FPP had no effect.

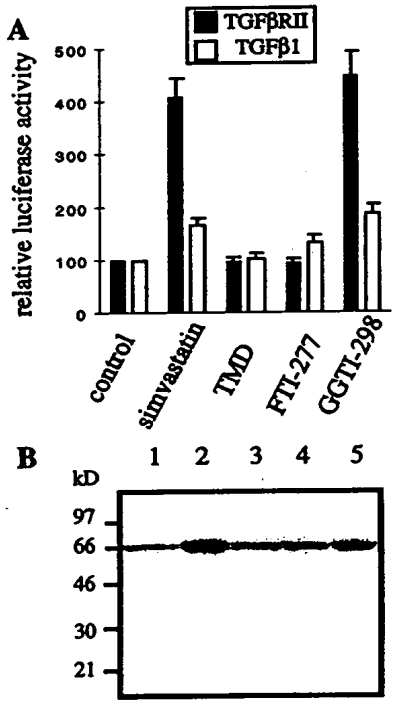

FIG. 3. Up-regulation of TGFβRII and TGFβ$_1$ expression by inhibition of the geranylgeranylation pathway. (*A*) Embryonic chicken atrial cells were transfected with either pTGFβRII-500/36-Lux or phTGFβ5-Lux plus pCMVβgal. Cells were allowed to recover followed by a 16-h incubation in media supplemented with LPDS and the various inhibitors. Lane 1, control; lane 2, 20 μM simvastatin; lane 3, 50 μM TMD; lane 4, 10 μM FTI-277; lane 5, 10 μM GGTI-298. Luciferase activity was normalized to β-galactosidase activity. Data are plotted as the mean ± SEM of three independent experiments. (*B*) Cells were grown in media with LPDS and treated with various inhibitors as described above. Thirty micrograms of crude cell extract were analyzed for the expression of TGFβRII protein using a TGFβRII antibody. Data are typical of three similar experiments.

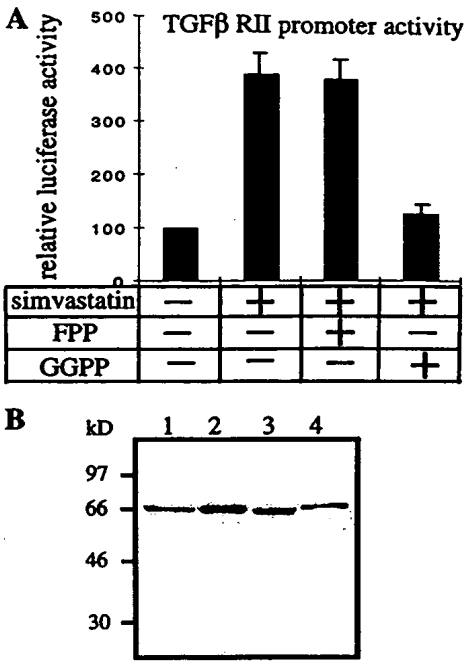

FIG. 4. GGPP reverses the effect of simvastatin on TGFβRII expression. (*A*) Embryonic chicken atrial cells were transfected with pTGFβRII-500/36-Lux plus pCMVβgal. After recovery, cells were incubated for 16 h in media with LPDS plus: lane 1, no additions; lane 2, 10 μM simvastatin; lane 3, 10 μM simvastatin plus 10 μM FPP; lane 4, 10 μM simvastatin plus 10 μM GGPP. Luciferase activity was normalized to β-galactosidase activity. Data are plotted as the mean ±SEM of four independent experiments. (*B*) Cells were grown in media supplemented with LPDS and treated as described above. Thirty micrograms of crude cell extract were analyzed by immunoblotting for the expression of TGFβRII protein. Data are typical of three similar experiments.

To determine the physiological significance of the coordinate up-regulation of TGFβRII and TGFβ$_1$ expression, we studied the effect of regulating the cholesterol metabolic pathway on PAI-1 promoter activity (32). Growth of cells in LPDS decreased PAI-1 promoter activity by 71 ± 2% (n = 4) compared with cells cultured with FCS, whereas simvastatin reversed the effect of LPDS on PAI-1 promoter activity (Fig. 5*A*). This effect of simvastatin was dose dependent; 0.2 μM simvastatin increased PAI-1 promoter activity by 31 ± 2% (n = 4) compared with cells grown in LPDS alone ($P < 0.01$), whereas 1 μM simvastatin increased PAI-1 promoter activity by 210 ± 8% (n = 4).

To determine whether the effect of simvastatin on PAI-1 promoter activity was also mediated via an effect on the geranylgeranylation pathway, we compared the effect of TMD, FTI-277, and GGTI-298 on PAI-1 promoter activity. GGTI-298 mimicked the effect of simvastatin and increased PAI-1 promoter activity up to 5.5 ± 0.48-fold (n = 4), whereas TMD and FTI-277 showed no effect (Fig. 5*B*). GGPP, but not FPP, completely reversed the effect of simvastatin on PAI-1 promoter activity (Fig. 5*B*). Hence TGFβ signaling is regulated by the cholesterol metabolic pathway in parallel with the regulation of TGFβRII and TGFβ$_1$ via an effect on protein geranylgeranylation.

If induction of the cholesterol metabolic pathway by growth of cells with LPDS interferes with TGFβ signaling by increasing the availability of GGPP, then addition of GGPP to cells grown in the presence of an exogenous source of cholesterol might also inhibit PAI-1 promoter activity. Incubation of cells in medium supplemented with FCS plus 10 μM GGPP resulted in a 56 ± 2% decrease (n = 3) in PAI-1 promoter activity

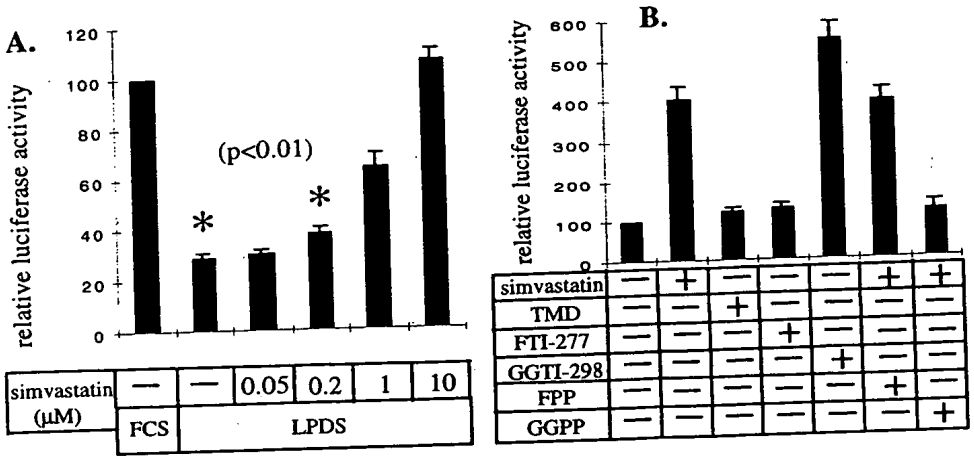

FIG. 5. Regulation of TGFβ signaling by control of the cholesterol metabolic pathway. Embryonic chicken atrial cells were transfected with p3TP-Lux plus pCMVβgal. After recovery, cells were incubated for 16 h in media supplemented with either (*A*) FCS, LPDS, or LPDS plus various concentrations of simvastatin (0–10 μM); (*B*) LPDS and either: no additions; 10 μM simvastatin; 50 μM TMD; 10 μM FTI-277; 10 μM GGTI-298; 10 μM simvastatin plus 10 μM FPP; 10 μM simvastatin plus 10 μM GGPP. Luciferase activity was normalized to β-galactosidase activity. Values are the mean ± SEM of four independent experiments.

compared with cells cultured in FCS alone (Fig. 6*A*). This decrease was similar to the effect of LPDS on PAI-1 promoter activity.

RhoA GTPase Regulates TGFβ Signaling. *Clostridium botulinum* C3 toxin specifically catalyzes the ADP ribosylation of members of the Rho family of small GTP-binding proteins and inhibits their function (33). Addition of C3 toxin to cultured embryonic chicken atrial cells resulted in a 2.7 ± 0.28-fold increase ($n = 3$) in TGFβRII promoter activity and a 4.5 ± 0.38-fold increase ($n = 3$) in PAI-1 promoter activity (Fig. 6*B*). These results implicate a member of the Rho family of small GTP-binding proteins in the regulation of TGFβRII and PAI-1 promoter activity.

To further establish which member of the Rho family played a role in the regulation of TGFβ signaling, cells were cotransfected with a PAI-1 promoter reporter and a vector expressing either a dominant-active RhoA mutant, a dominant-negative RhoA mutant, or a vector containing the C3 exotoxin gene. Each of these constructs was tagged at the N terminus with a myc-epitope. Cells transfected with the dominant-active mutant of RhoA demonstrated a 37 ± 9% decrease ($n = 3$) in PAI-1 promoter activity compared with control ($P < 0.01$). Cotransfection with a dominant-negative mutant resulted in a 5.8 ± 0.62-fold increase ($n = 3$) in PAI-1 promoter activity whereas cotransfection with C3 toxin resulted in a 4.8 ± 0.76-fold ($n = 3$) increase (Fig. 7*A*). Data in Fig. 7*B* demonstrate that each of these genes was expressed in cultured chicken atrial cells as detected by immunoblot analysis by using an anti-myc antibody. These results indicate that RhoA GTPase may regulate TGFβ signaling.

DISCUSSION

Several studies have suggested a possible relationship between $TGF\beta_1$ expression and cholesterol metabolism. An increase in expression of $TGF\beta_1$ has been observed in balloon-injured carotid arteries of rabbits treated with the HMGCoA reductase inhibitor NK-104, while the HMGCoA reductase inhibitor lovastatin decreased $TGF\beta_1$ expression in glomeruli of diabetic rats (21, 22). Furthermore, cholesterol feeding has been shown to effect $TGF\beta_1$ expression in aortas of Watanabe rabbits (8). These results appear to be cell-type specific and do not demonstrate an effect on TGFβ signaling. Data presented here extend these studies by demonstrating a relationship between cholesterol metabolism and TGFβ signaling. The induction of the cholesterol metabolic pathway by growth of embryonic chicken atrial cells in the absence of lipoproteins resulted in a coordinate decrease in the expression of TGFβRII, $TGF\beta_1$, and TGFβ signaling as measured by a decrease in PAI-1 promoter activity. Conversely, inhibition of the cholesterol metabolic pathway by the hydrophobic HMGCoA reductase

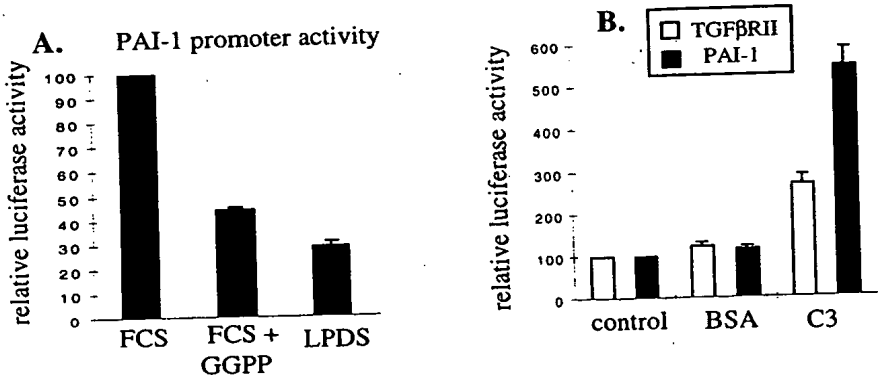

FIG. 6. Regulation of TGFβ signaling via the geranylgeranylation pathway. (*A*) Cells were transfected with p3TP-Lux plus pCMVβgal. After recovery, cells were incubated for 16 h in media with: lane 1, FCS alone; lane 2, FCS plus 10 μM GGPP; lane 3, LPDS alone. Luciferase activity was normalized to β-galactosidase activity. Data are plotted as the mean ± SEM of three independent experiments. (*B*) Cells were transfected with either pTGFβRII-500/36-Lux or p3TP-Lux plus pCMVβgal. After recovery, cells were cultured for 16 h in media supplemented with LPDS plus: lane 1, control; lane 2, 50 μg BSA/ml; lane 3, 50 μg C3 exotoxin/ml. Luciferase activity was normalized to β-galactosidase activity. Values are the mean ± SEM of three independent experiments.

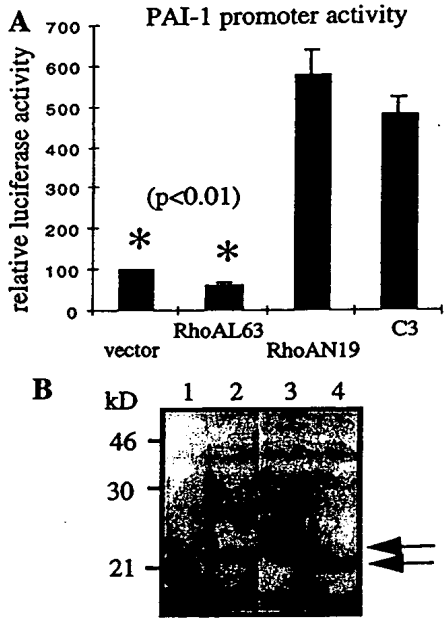

FIG. 7. Regulation of TGFβ signaling by RhoA GTPase. (*A*) Embryonic chicken atrial cells were cotransfected with p3TP-Lux, pCMVβgal, and either pCDNA3, pRK5 myc-RhoA L63, pCDNA3 myc-RhoA N19, or pEFmyc-C3. After recovery, cells were cultured for 16 h in media with LPDS. Luciferase activity was normalized to β-galactosidase activity. Values are the mean ± SEM of three independent experiments. (*B*) Thirty micrograms of crude cell extract from cells were analyzed for the expression of RhoA mutants and C3 toxin by immunoblotting using a myc antibody. Data are typical of three similar experiments.

inhibitors markedly increased the expression of TGFβRII, $TGF\beta_1$, and TGFβ signaling.

Geranylgeranylation is required for the membrane localization and functioning of small GTP-binding proteins such as Rho family members. Previous data have demonstrated that posttranslational lipidation might be a regulatable process. Thus induction of the cholesterol metabolic pathway was capable of increasing the farnesylation and membrane localization of Ras (12). The finding that GGPP, the substrate for geranylgeranyltransferase, not only reversed the effect of simvastatin on TGFβ signaling, but also mimicked the effect of induction of the cholesterol metabolic pathway on TGFβ signaling in cells cultured in FCS, supports the conclusion that increased substrate availability for geranylgeranyltransferase caused by either induction of the cholesterol metabolic pathway or exogenously added GGPP inhibits the expression of TGFβRII and $TGF\beta_1$.

The finding that overexpression of a dominant-active RhoA mutant mimicked the effect of LPDS (induction of the cholesterol metabolic pathway) on TGFβ signaling and a dominant-negative RhoA mutant mimicked the effect of HMGCoA reductase inhibitors on TGFβ signaling supports the conclusion that RhoA GTPase is the geranylgeranylated protein that negatively regulates TGFβ signaling. Atfi *et al.* and Musci *et al.* demonstrated that the response of the PAI-1 promoter to exogenously added TGFβ was inhibited by dominant-negative mutants of Rho family members, suggesting that Rho positively regulated TGFβ signaling (34, 35). Our studies, carried out in the absence of exogenously added TGFβ, measured the effects of regulating the cholesterol metabolic pathway on the function of an autocrine loop for TGFβ signaling. In contrast to those studies, our data support the existence of a negative control of TGFβ signaling by RhoA. These differences could be cell-type specific. Taken together, these data suggest a new mechanism for the control of $TGF\beta_1$ and TGFβRII expression and TGFβ signaling via the regulation of RhoA GTPase function.

Prior studies have suggested that TGFβ regulates the cholesterol metabolic pathway via an effect on the expression of LDL receptors (6, 7). In bovine adrenocortical cells, TGFβ interfered with steroidogenesis in parallel with a decrease in the level of LDL receptors (36). TGFβ has also been shown to stimulate the expression of LDL receptors in HepG2 cells and human mesangial cells and decrease the incorporation of [^{14}C]acetate into cholesterol (7, 37). Because GGPP and FPP are products of the cholesterol metabolic pathway, a decrease in endogenous cholesterol production because of TGFβ induction of LDL receptor number should decrease GGPP and FPP levels. Hence, TGFβ might be expected to interfere with the geranylgeranylation and farnesylation of small GTP-binding proteins by an effect on substrate availability. In support of this conclusion, recent studies from our laboratory demonstrate that in cultured embryonic chicken atrial cells, TGFβ regulates the lipidation of Ras by FPP (S. M. Ward and J.B.G., unpublished results). Hence, if as suggested by the data presented here a Rho family member regulates TGFβ signaling, and if TGFβ signaling regulates availability of substrates for the lipidation of small GTP-binding proteins, a feedback loop might exist by which TGFβ might regulate its own expression via control of protein geranylgeranylation.

Recent studies have demonstrated that HMGCoA reductase inhibitors decrease coronary events in patients suffering from coronary artery disease (17, 18). Further analysis has suggested that cholesterol reduction alone does not appear to fully account for the decrease in coronary events (38). The finding of a relationship between the regulation of cholesterol metabolism and TGFβ signaling suggests that alterations in TGFβ signaling in response to HMGCoA reductase inhibitors may be responsible for some of the therapeutic effects of these agents.

The finding that HMGCoA reductase inhibitors, simvastatin and atorvastatin, but not pravastatin, are capable of inducing an increase in the expression of TGFβRII could have important implications for the mechanism of action of these agents. Although HMGCoA reductase inhibitors are structurally quite similar, they differ markedly in hydrophobicity: simvastatin > atorvastatin > pravastatin (19, 20, 39). Although all three of these agents are transported into the liver, uptake into nonliver cells depends on relative hydrophobicity. Hence it is likely that differences in hydrophobicity are responsible for the finding that only simvastatin and atorvastatin effected the expression of TGFβRII in cultured heart cells. Therapeutic doses of simvastatin and pravastatin have been shown to result in serum levels of 0.02–0.27 μM and 0.09–0.16 μM, respectively (40). These concentrations are similar to those found to have significant effects on TGFβ signaling in cultured chicken atrial cells reported here.

Based on the data presented here, the effects of cholesterol-lowering therapy on TGFβ signaling might be expected to differ depending on whether patients are treated by the dietary restriction of cholesterol, a hydrophilic HMGCoA reductase inhibitor such as pravastatin, or hydrophobic HMGCoA reductase inhibitors such as simvastatin or atorvastatin. Although the role of TGFβ in atherogenesis remains controversial, differences in effects on TGFβ signaling could be important clinical distinctions between different classes of HMGCoA reductase inhibitors and different modes of cholesterol-lowering therapy.

PAI-1 plays a role in the inhibition of thrombolysis and smooth muscle cell migration and in increasing the stability of atherosclerotic plaques (41). Thus a change in PAI-1 expression in response to HMGCoA reductase inhibitors could have important clinical implications. A number of studies have attempted to determine the effect of HMGCoA reductase inhibitors on levels of PAI-1 in hypercholesterolemic patients. Patients treated with simvastatin and atorvastatin demon-

ated an increase in PAI-1 activity, whereas pravastatin atment was associated with a decrease in PAI-1 (20, 42, 43). ecent study using SV40-transformed rat aortic endothelial s demonstrated a decrease in PAI-1 activity in response to astatin (44). These effects of HMGCoA reductase inhibi- s are likely to depend on cell type and cell density and may different in transformed cells.

Both TGFβ and HMGCoA reductase inhibitors have been wn to interfere with cell division and cellular migration (25, 46). HMGCoA reductase inhibitors have been shown to uce the expression of $p21^{WAF1/C1P1}$, which negatively regulates -cycle progression by inhibiting cyclin-dependent kinase ac- y (25, 46). Both HMGCoA reductase inhibitors and GGTI- were shown to regulate $p21^{WAF1/C1P1}$ expression via an effect a TGFβ response element in the upstream region of the $^{WAF1/C1P1}$ promoter (46, 47). Taken together with our data demonstrate that both simvastatin and GGTI-298 increase Fβ signaling, these data support the conclusion that HMG- A reductase inhibitors regulate $p21^{WAF1/C1P1}$ expression and cell cycle via the stimulation of TGFβ signaling.

he role of an increase in TGFβ signaling in cardiomyocytes esponse to HMGCoA reductase inhibitors is unclear. Fβ signaling has been associated with cardiac hypertrophy, early response to myocardial infarction, ventricular remod- g, and hypertrophic cardiomyopathy (1–2). Hypertrophic iomyopathy has been associated with an increase in $Fβ_1$ expression and in the number of TGFβ receptors (48). pressure-loaded model for cardiac hypertrophy in which were subjected to abdominal aortic constriction or sub- neous norepinephrine, cardiac myocytes derived from the ts of these animals demonstrated an increase in $TGFβ_1$ In acute myocardial infarction 24–48 hr after ligation of eft coronary, artery levels of $TGFβ_1$ mRNA increased 2- fold compared with controls (2). Our data suggest the guing hypothesis that treatment with the more hydropho- IMGCoA reductase inhibitors might exert an effect on the ogenesis of these processes and contribute to their pro- ion by stimulating the TGFβ signaling pathway.

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A RhoA-derived peptide inhibits syncytium formation induced by respiratory syncytial virus and parainfluenza virus type 3

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The fusion glycoproteins of human respiratory syncytial virus (RSV) and human parainfluenza virus type-3 (PIV-3) mediate virus entry and syncytium formation. Interaction between the fusion protein of RSV and RhoA, a small GTPase, facilitates virus-induced syncytium formation. We show here a RhoA-derived peptide inhibits RSV and syncytium formation induced by RSV and PIV-3, both *in vitro* by inhibition of cell-to-cell fusion and *in vivo* by reduction of peak titer by 2 $\log_{10}$ in RSV-infected mice. These findings indicate that the interaction between these two paramyxovirus fusion proteins and RhoA is an important target for new antiviral strategies.

Human respiratory syncytial virus (RSV) and parainfluenza virus type-3 (PIV-3) belong to the *Paramyxoviridae* family. RSV and PIV-3 are the main viral causes of acute lower respiratory tract illness in infants[1,2]. There is no effective antiviral therapy or vaccine available for either virus.

RSV and PIV-3 each have two main surface envelope glycoproteins: an attachment protein (RSV G or PIV-3 hemagglutinin–neuraminidase) and a fusion protein (F). The G or hemagglutinin–neuraminidase glycoproteins are thought to mediate virus attachment to the cell receptor[3,4]. The receptor for PIV-3 is sialic acid[4], and RSV G may bind to heparan sulfate[5,6]. The F glycoprotein mediates at least two essential steps in the virus life cycle that require membrane fusion: it promotes fusion of the viral and cellular membranes with subsequent transfer of viral genome material into the cell, and promotes fusion of the infected cell membrane with those of adjacent cell membranes, leading to syncytia formation. The F glycoproteins of RSV and PIV-3 are synthesized as inactive precursors, which are co-translationally modified by the addition of N-linked glycosylation in the endoplasmic reticulum. The F precursor is cleaved by cellular trypsin-like endoproteases into two disulfide-linked subunits, F1 and F2, before reaching the cell surface, and is assembled as a higher-order homooligomer[7,8]. RSV can infect cells *in vitro* despite lacking the G glycoprotein[9], indicating that F may have additional functions of attachment to a host co-receptor.

The process of membrane fusion is essential for the entry of both cell-free and cell-associated virus. RSV and PIV-3 share the properties of syncytium formation and pH-independent fusion from without. Common host determinants may be involved in the essential processes required for viral entry and syncytium formation. The fusion glycoproteins of these viruses share elements of similar function, structure and, in some cases, sequence, indicating a common membrane fusion mechanism[10]. In particular, paramyxovirus F proteins share conserved hydrophobic sequences at their amino termini, important for interactions with the lipid bilayer, followed by a region of heptad repeats[10].

Although much is known about the properties of membrane fusion in selected cellular compartments or in artificial lipid membranes, the molecular mechanisms for both virus-induced and cellular fusion reactions remain mostly undefined[11]. Cell-to-cell fusion mediated by some viral envelope proteins involves cellular actin cytoskeleton and cell surface integrins[12,13]. Host cellular proteins that maintain cell membrane integrity and cell mobility, such as RhoA, might be expected to be involved in virus-induced fusion and syncytium formation; however, there is no direct evidence at present for their involvement.

RhoA binds to RSV F protein and mediates virus-induced syncytium formation[14]. In addition, RhoA amino acids 67–110 bind to RSV F amino acids 146–155. RhoA, a small GTPase of the Ras superfamily, controls a plethora of biological functions including actin reorganization, gene expression, cell morphology, cell motility and cell proliferation[15]. We report here a RhoA peptide derived from the domain that binds the RSV F protein has potent antiviral activity against RSV and PIV-3 infection in cell culture and in RSV-infected mice. These data provide additional evidence for conserved structural and functional features between the fusion proteins of RSV and PIV-3 and for the possibility of common mechanisms involving virus-induced membrane fusion. The inhibitory effect of this $RhoA_{77\text{-}95}$ peptide may be useful for the development of new antiviral strategies with broad application.

Effects of RhoA peptides on RSV-induced syncytium

We synthesized three overlapping 19-amino-acid peptides spanning the F binding domain of RhoA (amino acids 67–110) and used these to study the effect of peptides on virus-induced syncytium formation. Pretreatment of virus inoculum with the $RhoA_{77\text{-}95}$ peptide completely blocked plaque formation, whereas pretreatment with $RhoA_{87\text{-}105}$ did not have any effect on the plaque formation relative to media-treated controls (Fig. 1). The $RhoA_{67\text{-}85}$ peptide did not block RSV-induced syncytium forma-

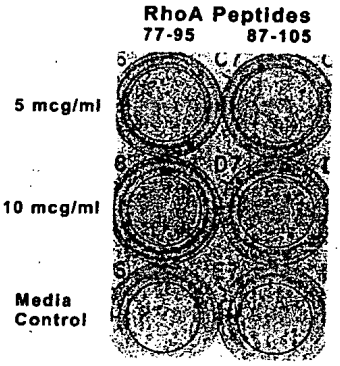

Fig. 1 RhoA-derived peptide inhibits RSV infection and syncytium formation. Addition of $RhoA_{77-95}$ (left) but not $RhoA_{87-105}$ (right) to RSV stock[18] prevents plaque formation in HEp-2 cells.

tion. Varying the concentration of $RhoA_{77-95}$ showed that the concentration required to inhibit number of plaques by 50% (IC_{50}) was 0.54 μg/ml, or 0.25 μM. Immunoperoxidase staining did not show any RSV antigen-positive cells treated with $RhoA_{77-95}$, indicating that RSV replication was inhibited at a step before viral protein synthesis. These data indicate that the $RhoA_{77-95}$ peptide inhibits RSV at an early step of the replication cycle.

We next determined whether the $RhoA_{77-95}$ peptide could inhibit cell-to-cell spread of RSV after infection. We used a recombinant RSV (rgRSV) expressing a gene for green fluorescent protein, located at the first position in RSV gene order. We added 5 μg/ml $RhoA_{77-95}$ to cells 0, 4 and 24 hours after RSV adsorption, and evaluated HEp-2 monolayers by fluorescent microscopy 48 hours after infection (Fig. 2). There was inhibition of cell-to-cell spread and syncytia formation in rgRSV-infected cells treated with the $RhoA_{77-95}$ peptide compared with that of untreated, infected cells. The number of infected cells increased between 4 and 24 hours after infection, indicating the completion of one round of viral replication, production of new virions and infection of new cells before 24 hours. Therefore, the addition of $RhoA_{77-95}$ seems to prevent subsequent spread of cell-free virus to other cells as well as preventing syncytium formation. These data also support the possibility of a block at an early step in the viral life cycle.

Next, we tested whether $RhoA_{77-95}$ peptide inhibition of RSV was reversible. We dialyzed RSV–$RhoA_{77-95}$ peptide, RSV–$RhoA_{87-105}$ peptide and RSV–media suspensions overnight and added these to HEp-2 cell monolayers. There were no RSV plaques in wells treated with dialyzed RSV–$RhoA_{77-95}$ peptide suspension, in contrast to the presence of many plaques in wells treated with dialyzed RSV–$RhoA_{87-105}$ peptide or RSV–media suspensions (data not shown). This indicates that the $RhoA_{77-95}$ peptide binds with high avidity to RSV F.

Cell-to-cell fusion assay

To determine whether the $RhoA_{77-95}$ and $RhoA_{87-105}$ peptides have biological effects in cell-to-cell fusion induced by RSV F, we used an assay based on the cytoplasmic activation of the reporter gene β-galactosidase. The RSV envelope proteins F, G and SH have been shown in this assay to optimize cell-to-cell fusion[16,17]. We did this assay as described[16] with some modifications. One cell population was infected with the recombinant vaccinia virus vTF7-3 and transfected with three separate plasmids containing RSV F, G and SH genes. The other cell population was infected with a recombinant vaccinia virus expressing β-galactosidase. The two cell populations were mixed and either treated with 5 μg/ml peptides or left untreated. Cell fusion was measured by an *in situ* assay using X-gal staining of cells (Fig. 3*a–c*) or a quantitative colorimetric lysate assay for β-galactosidase (Fig. 3*d*). Wells treated with the $RhoA_{77-95}$ peptide showed no blue fused cells (Fig. 3*b*), in contrast to wells treated with the $RhoA_{87-105}$ peptide (Fig. 3*c*) or untreated wells (Fig. 3*a*). The *in situ* assay correlated with the quantitative colorimetric assay for the treated and untreated wells (Fig. 3*d*). Control wells containing one population of cells expressing G and SH and another population of cells infected with vaccinia virus expressing β-galactosidase did not show β-galactosidase activity (data not shown). These data show that the cell-to-cell fusion induced by RSV F was blocked by $RhoA_{77-95}$ peptide, and indicate that the cell-to-cell spread of virus through syncytium formation may require the binding of F to RhoA.

$RhoA_{77-95}$ peptide inhibits RhoA–RSV F interaction

To determine whether the $RhoA_{77-95}$ peptide can interfere with the interaction between F and RhoA, we did a competitive enzyme-linked immunosorbent assay (ELISA) (Fig. 4). We coated wells with 25 ng purified F, and after blocking, added 50 ng RhoA and increasing concentrations of peptide (range, 100 ng/ml to 500 μg/ml), then detected bound RhoA with monoclonal antibody against RhoA. We used RhoA without the peptide as a positive control. As a negative control, we used Rac1 protein and $RhoA_{87-105}$ instead of RhoA and $RhoA_{77-95}$, respectively. There was a correlation between concentration of $RhoA_{77-95}$ peptide and inhibition of RhoA interaction with F. The results for all concentrations of $RhoA_{87-105}$ were similar to data from the untreated positive control, whereas the results for Rac1 binding to F were similar to background values. These data indicate that the biologic effects demonstrated with the peptide in cell culture and *in vivo* are based on its inhibition of the interaction between RhoA and the viral fusion protein.

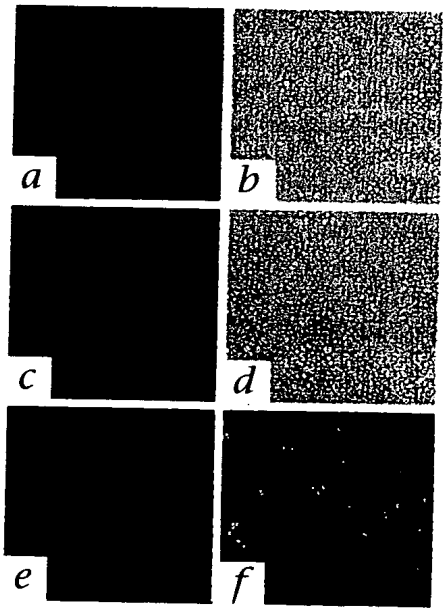

Fig. 2 Effect of $RhoA_{77-95}$ added after rgRSV infection. Syncytium formation was assessed 48 h after infection. ***a*** and ***b***, Peptide added 0 h after infection. *b*, Phase contrast image of *a*. ***c*** and ***d***, Peptide added 4 h after infection. *d*, Phase contrast image of *c*. ***e***, Peptide added 24 h after infection. ***f***, No peptide added.

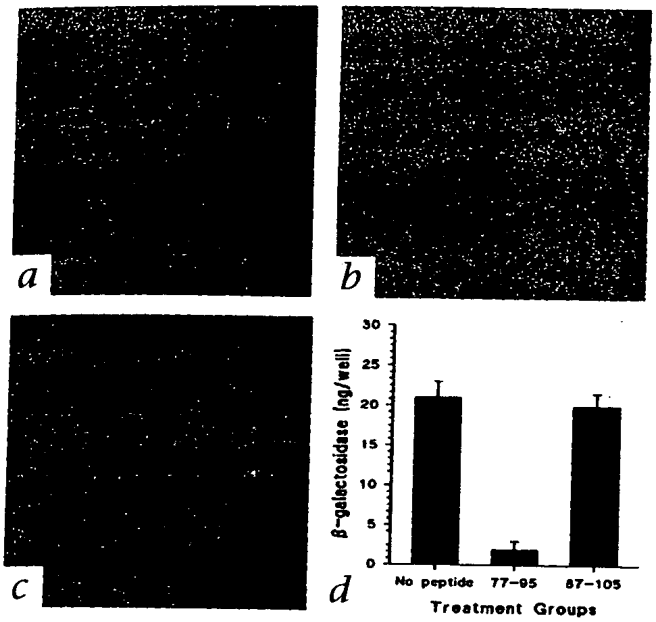

Fig. 3 Effect of RhoA-derived peptides on cell-to-cell fusion. HEp-2 cells expressing RSV F, G, and SH glycoproteins were untreated (*a*) or treated with $RhoA_{77-95}$ (*b*) or $RhoA_{87-105}$ (*c*), then mixed with another cell population infected with recombinant vaccinia virus expressing β-galactosidase, then assessed by *in situ* X-gal staining (***a***, ***b*** and ***c***) or quantitative colorimetric lysate assay (*d*). ***d***, Equal volumes of cell lysates and 2× substrate solution were mixed and the rates of substrate hydrolysis was monitored by measuring absorbance at 590 nm with a spectrophotometer. The data are presented as β-galactosidase (ng/well) produced by the cells. Each point represents an average of three experiments ± standard deviation.

Next, we determined whether the binding of $RhoA_{77-95}$ peptide to F was reversible or nonreversible in the ELISA. We added RhoA protein 1 hour after allowing $RhoA_{77-95}$ peptide to bind F protein, incubated this for 1 hour, and detected RhoA using monoclonal antibodies against RhoA. There was no binding of RhoA to F (data not shown). Thus, the $RhoA_{77-95}$ peptide binds F with high avidity.

$RhoA_{77-95}$ peptide can block infection in an animal model for RSV

To determine whether the RhoA peptide would alter the course of infection *in vivo*, we infected BALB/c mice intranasally with 0.1 ml containing 10^7 plaque-forming units (PFU) live RSV, using a well-established model[18]. We administered 500 μg $RhoA_{77-95}$ peptide, $RhoA_{87-105}$ peptide or PBS intranasally immediately before or 2 hours or 4 days after RSV infection. Mice treated with the $RhoA_{77-95}$ peptide immediately before or 2 hours after virus infection had no discernable illness or weight loss, whereas PBS-treated control mice experienced a typical illness pattern (Fig. 5*a*) with a 22% peak weight loss from baseline. Mice treated with the peptide 4 days after virus infection had illness or weight loss similar to that of mice treated with PBS. Plaque assays of lungs on day 4 after RSV challenge showed that peptide treatment immediately before or 2 hours after virus infection reduced RSV titers by 2 $\log_{10}$ compared to the RSV titers in lungs from control mice treated with PBS or the $RhoA_{87-105}$ peptide (Fig. 5b). These data indicate that the diminished illness in mice treated with the $RhoA_{77-95}$ peptide before RSV infection could be due to inhibition of virus replication by the peptide.

$RhoA_{77-95}$ peptide can inhibit infection by PIV-3

As the amino terminus of the RSV fusion glycoprotein is similar in structure and hydrophobicity to that of other enveloped viruses[10], we next determined whether the $RhoA_{77-95}$ peptide could block infection with PIV-3, influenza A virus (H3N2 A/Beijing/1996), herpes simplex virus type I, vaccinia virus or a coronavirus (mouse hepatitis virus strain A59). Syncytium formation was inhibited in PIV-3-infected HEp-2 cells (Fig. 6) by the $RhoA_{77-95}$ peptide. However, infection with influenza virus, herpes simplex virus type I, vaccinia virus, or coronavirus was not inhibited by the $RhoA_{77-95}$ peptide (data not shown). Treatment of PIV-3 with the $RhoA_{87-105}$ peptide had no effect on syncytium formation (Fig. 6). These data indicate that a common mechanism of virus-induced syncytium formation involving RhoA may be shared between these two paramyxoviruses. They also indicate that although a common motif is present at the amino terminus in the fusion proteins of many enveloped viruses, host cell components involved in syncytium formation may differ between virus families.

Discussion

The hallmark of the RSV and PIV-3 cytopathic effect in cell culture is extensive syncytium formation. The fusion glycoprotein (F) mediates virus-induced fusion at neutral pH, and for PIV-3, membrane fusion depends on the density of the fusion protein on the infected cell surface[19]. Cellular proteins contributing to paramyxovirus-induced syncytia formation have not been identified before. However, the lipid composition and other properties of the target cell membrane can influence the ability of a virus to produce syncytia[20,21]. A host protein, RhoA, has been shown to bind to viral fusion glycoprotein and facilitates syncytium formation[14]. We have shown here the inhibition of RSV- and PIV-3-induced syncytium formation by a RhoA-derived peptide ($RhoA_{77-95}$) from the RSV F binding domain. This inhibition indicates that two viruses from the *Paramyxoviridae* family may use the same host cell protein and same binding site on this protein in the process of inducing cell-to-cell fusion, strengthening the concept of conserved functional and structural features of paramyxovirus fusion proteins. The role of RhoA in virus-induced fusion is not yet defined and might include a direct effect, or an indirect effect through signaling events on cellular cytoskeletal organization, cell shape or cell motility. The F protein interaction with RhoA might also be involved in other aspects

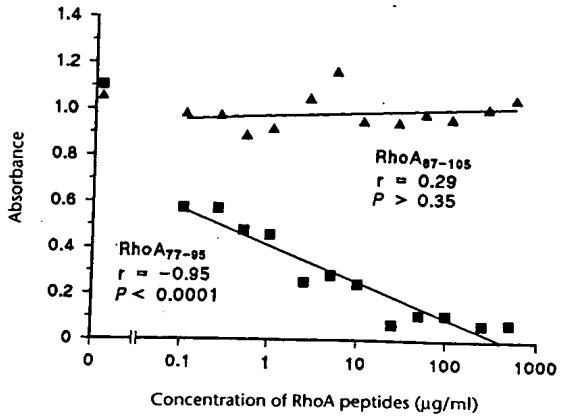

Fig. 4 $RhoA_{77-95}$ peptide competitively inhibits RhoA interaction with RSV F in an ELISA. Wells were coated with purified F protein and blocked, and RhoA was added in the presence of $RhoA_{77-95}$ (■) or $RhoA_{87-105}$ (▲) peptide (horizontal axis, increasing concentrations). Bound RhoA was detected by monoclonal antibodies against RhoA.

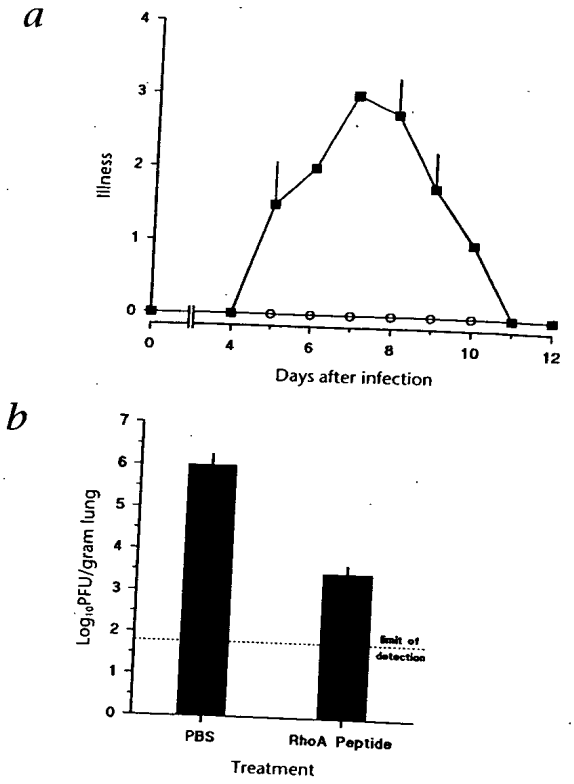

Fig. 5 $RhoA_{77-95}$ peptide treatment reduces illness and viral titers in RSV-infected mice. ***a***, Mean clinical illness scores[18] in RSV-infected mice treated with peptide (○) or PBS (■) on day 0 immediately before RSV infection. Error bars represent standard deviations. ***b***, RSV titers in lung on day 4 after challenge. Mice were treated with PBS (left) or the $RhoA_{77-95}$ peptide (right) immediately before RSV infection on day 0. Data represent geometric means with standard deviations (error bars). These are the combined results of two independent experiments with a total of 10 mice in each group. pfu, plaque-forming unit.

of virus replication including assembly, filament formation or budding.

Peptides derived from the heptad repeat region of viral fusion protein[22–25] inhibit syncytium formation by homologous virus by binding to the heptad repeats interfering with fusion protein structure. The inhibitory peptide described here is of a different nature. We have shown here that a peptide derived from a cellular protein that does not have sequence homology to the viral fusion proteins can specifically inhibit virus-induced syncytium formation and possibly virus entry. In a yeast two-hybrid assay, RhoA was shown to not bind a RSV F construct expressing the heptad repeat region of the fusion proteins (F_{N155} construct encoding F residues 155–550)(ref. 14), indicating that the mechanism of fusion inhibition by the RhoA-derived peptide is different from that for peptides interfering with the heptad repeat interactions.

The complete inhibition of RSV infection when the $RhoA_{77-95}$ peptide was added before RSV adsorption to cells (Fig. 1) or immediately after rgRSV infection (Fig. 2*a*) indicates that the peptide interferes with virus-mediated fusion events. Immunohistochemical staining for RSV F (Fig. 1) showed no expression of RSV F in HEp-2 cells. Analysis of the virus growth curve indicated that there was no virus yield (data not shown). Peptide inhibition of green fluorescent protein expression in rgRSV-infected cells indicated that the $RhoA_{77-95}$ peptide interfered at an early step in the virus replication pathway, possibly at entry. The same could be true with PIV-3 infection (Fig.6). The inhibitory effect of $RhoA_{77-95}$ peptide was specific for the amino-acid sequence of the synthetic peptide, as a $RhoA_{77-95}$ peptide analog with the same amino acid content but random sequence did not inhibit RSV-induced syncytium formation (data not shown). This also indicates that the inhibitory effect of the $RhoA_{77-95}$ peptide is not based on hydrophobicity or charge.

The role of RhoA in cell-to-cell fusion is supported by the syncytium-inhibiting effect of $RhoA_{77-95}$ peptide in a RSV-free cell-to-cell fusion assay (Fig. 3). Membrane fusion involves the mixing of both membrane lipid layers, resulting in the mixing of the aqueous contents of donor and recipient cells. In our fusion assay, quantitation of the reporter gene (β-galactosidase) activation demonstrates the content mixing potential of the RSV F protein in the presence or absence of peptides. Cell-to-cell fusion was not inhibited by $RhoA_{87-105}$ peptide, but $RhoA_{77-95}$ peptide inhibited content mixing by preventing cell-to-cell fusion. This result is consistent with the data from rgRSV-infected HEp-2 cells in the presence of RhoA peptides (Fig. 2), in which the $RhoA_{77-95}$ peptide inhibited not only virus–cell fusion but also virus-induced cell-to-cell fusion.

The data from competitive ELISA strongly indicate that the biological effects of the $RhoA_{77-95}$ peptide are based on interference with the interaction between RhoA and the viral fusion proteins. The high avidity binding of the $RhoA_{77-95}$ peptide to F supports prior analysis of the RSV F–RhoA interaction by biomolecular interaction analysis based on surface plasmon resonance. This observation was also supported by data obtained from the dialysis experiment, in which the $RhoA_{77-95}$ peptide irreversibly prevented RSV-induced syncytium formation despite overnight dialysis.

Blockade of RSV and PIV-3 syncytium formation by the same peptide, $RhoA_{77-95}$ (Figs. 1 and 6, respectively), indicates that the mechanism of virus-induced membrane fusion is similar in both viruses. This provides additional evidence for conserved structural and functional features between the fusion proteins of RSV and PIV-3 indicated by sequence analysis[10], and raises the possibility of designing a single antiviral agent against both these paramyxovirus-induced diseases. For example, a RhoA-derived peptide ($RhoA_{77-95}$), a related peptidomimetic compound or a small molecule capable of interfering with the RhoA-fusion protein interaction could potentially have a clinical effect in RSV treatment similar to that of T-20, a peptide derived from the C-terminal heptad repeat of gp41, in HIV-1 treatment[26]. The inhibitory effect of $RhoA_{77-95}$ *in vivo* on virus replication and potential for diminishing illness in the mouse model of RSV (Fig. 5) indicate that early treatment with a RhoA-derived peptide has therapeutic potential in RSV or PIV-3 infections, particularly in giant cell pneumonia, seen in patients with bone marrow trans-

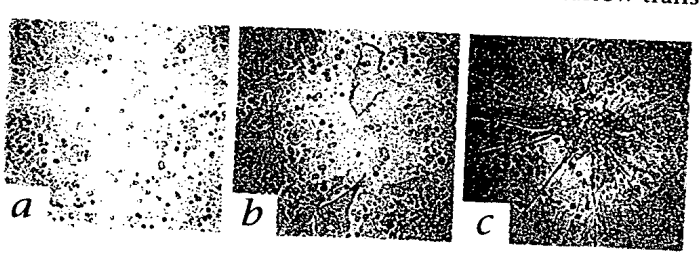

Fig. 6 Inhibition of PIV-3 infection by $RhoA_{77-95}$ peptide. ***a*** and ***b***, Peptides $RhoA_{77-95}$ (*a*) or $RhoA_{87-105}$ (*b*) were incubated with PIV-3, then the suspension was added to HEp-2 cells. ***c***, PIV-3-infected HEp-2 cells.

plantation, lung transplantation or severe combined immunodeficiency. Although the peptide treatment immediately before or 2 hours after RSV infection prevented illness in mice, treatment on day 4 after RSV infection had no effect on the illness (data not shown). This is consistent with other antiviral approaches and reflects the fact that when illness is mediated by the T-cell response and is not direct virus-induced cytopathology, antiviral therapy must be given early or combined with immunomodulators.

In conclusion, a RhoA-derived peptide ($RhoA_{77-95}$) was shown here to inhibit RSV and PIV-3 infection and syncytium formation. $RhoA_{77-95}$ also blocked cell-to-cell-fusion in a virus fusion glycoprotein (F)-induced cell-to-cell fusion assay. Given immediately before infection or 2 hours after RSV infection, the $RhoA_{77-95}$ peptide reduced RSV titer and prevented illness in mice. Exploiting the interaction between RhoA and F protein may also be useful for designing peptidomimetic or low-molecular-weight antiviral drugs.

Methods

Virus and cells. The A2 strain of RSV was provided by R. Chanock (National Institutes of Health, Bethesda, Maryland). The rgRSV was provided by M. Peeples (Rush Medical College, Chicago, Illinois) and P. Collins (National Institutes of Health, Bethesda, Maryland). Wild-type parainfluenza virus type 3 (PIV-3), influenza A virus (H3N2 A/Beijing/1996), herpes simplex virus type I and the coronavirus, mouse hepatitis virus strain A59 strains were provided by B. Murphy, P. Wright, P. Spearman and M. Denison, respectively. RSV stocks were prepared as described[18]. HEp-2 cells were maintained in Eagle's minimal essential media (EMEM) supplemented with glutamine, gentamicin, penicillin G and 10% fetal bovine serum (FBS).

RhoA-derived peptides. The peptides were synthesized by Research Genetics (Huntsville, Alabama) as the free acid, at approximately 70% purity in the desalted, lyophilized form. The major species in each preparation had the calculated molecular weight of the desired peptide, based on mass spectrometry. The amino-acid sequence of the peptides are: $RhoA_{67-85}$, DRLRPLSYPDTDVILMCFS; $RhoA_{77-95}$, TDVILMCFSIDSPDSLENI; $RhoA_{87-105}$, DSPDSLENIPEKWTPEVKH; and $RhoA_{77-95\ Scrambled}$, DDMSVISELICTSPLDFIN. All the peptides were resuspended in deionized distilled water.

Blockade of RSV infection in cell culture with peptides. For the assay, the peptides were incubated for 1 h on ice with 1×10^3 PFU/ml RSV at peptide concentrations of 5 and 10 μg/ml of media, then 100 μl of the suspension was added to HEp-2 cells in 96-well plates. For defining the IC_{50}, concentrations of peptide ranging from 0.1 μg/ml to 5 μg/ml were used. After 3 d, plates were fixed with methanol and RSV-specific immunoperoxidase staining was done[27]. RSV plaques were counted in each well.

For experiments using rgRSV, HEp-2 cells in 12 well plates were infected with 100 μl rgRSV stock virus. The $RhoA_{77-95}$ peptide (5 μg/ml) was added at 0, 4 and 24 h after infection. At 48 h after infection, infected cells were viewed by fluorescent microscopy. Cells infected with rgRSV without the peptide were used as a control.

For testing whether the peptide binding to RSV was reversible, 1 ml media containing 1×10^8 PFU/ml RSV and 100 μg/ml peptide ($RhoA_{77-95}$ or $RhoA_{87-105}$) suspension was incubated on ice for 1 h. RSV without the peptide was used as a control. A cellulose ester membrane (Spectrum Medical Industries, Houston, Texas) with a 10,000-dalton molecular weight cutoff was used to dialyze RSV–peptide suspension in PBS at 4 °C overnight. After overnight dialysis, the suspension was added to HEp-2 cells in a 12-well plate. After 2 d, plates were fixed and stained as described above. RSV plaques were counted in each well.

Cell fusion assay using vaccinia virus-based expression of RSV envelope glycoproteins. The ability of the $RhoA_{77-95}$ peptide to inhibit RSV F-induced cell-to-cell fusion was assessed using a quantitative assay based on the cytoplasmic activation of reporter gene β-galactosidase[16]. One population of HEp-2 cells was infected with recombinant vaccinia virus vTF7-3, which encodes T7 polymerase, at a multiplicity of infection of 10 PFU per cell, and was transfected with plasmids encoding RSV glycoproteins F, G and SH under control of the T7 promoter (gifts from P. Collins, National Institutes of Health, Bethesda, Maryland) using LipofectAMINE (Life Technologies). At 5 h after transfection, the cells expressing viral envelope proteins were trypsinized, suspended in MEM containing 2.5% FBS to a density of 2×10^7 cells per ml, and incubated overnight at 32 °C. The cells were then washed and suspended in Opti-MEM (Life Technologies) at a concentration of 1×10^6 cells per ml. A second population of HEp-2 cells was infected with recombinant vaccinia virus expressing β-galactosidase under control of the T7 promoter (provided by E.A. Berger, National Institutes of Health, Bethesda, Maryland). At 5 h after infection, cells were trypsinized and finally suspended at a concentration of 1×10^6 cells per ml. The cell population expressing the viral glycoproteins was treated with 5 μg/ml $RhoA_{77-95}$ or $RhoA_{87-105}$ peptide or left untreated, and incubated for 30 min at 37 °C. The two cell populations were mixed in triplicate by adding 100 μl of each cell population to 96-well tissue culture plates, which were then incubated at 37 °C for 4 h. Cell fusion was measured by the quantitative colorimetric lysate assay for β-galactosidase or an *in situ* assay using X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; Life Technologies) staining of cells. In the colorimetric lysate assay, 5 μl 20% (volume/volume) Nonidet-P40 was added to each well, and the contents were mixed by pipetting. β-galactosidase activity was quantified at ambient temperature in 96-well flat-bottomed plates by mixing 50 μl of each lysate with 50 μl 2X substrate solution (16 mM CPRG (chlorophenol-red-β–D- galactopyranoside; Boehringer), 0.12 M $Na_2HPO_4.7H_2O$, 0.08 M $NaH_2PO_4.H_2O$, 0.02 M KCl, 0.002 M $MgSO_4.7H_2O$ and 0.01 M β-mercaptoethanol). The rate of substrate hydrolysis at ambient temperature was monitored by measuring the absorbance at 590 nm with a spectrophotometer. The quantity of β-galactosidase was calculated by comparing the hydrolysis rates for each sample with that obtained for a standard commercial preparation of *Escherichia coli* β-galactosidase (600 U/mg; Boehringer). β-galactosidase levels were expressed as nanograms per well. For the *in situ* assay, 20 μl 10X fixative solution (20% formaldehyde and 2% glutaraldehyde in PBS) was added to each well. The plates were incubated at 4 °C for 5 min. Without disturbing the settled cells, 0.15 ml medium was gently removed and replaced with 0.15 ml of 37 °C-equilibrated staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride and 1 mg/ml X-Gal, freshly diluted from a 40 mg/ml stock solution in dimethyl formamide). The plates were incubated overnight at 37 °C to allow complete staining. Blue-stained syncytia were viewed with an inverted phase-contrast microscope.

Competitive ELISA. Immunoaffinity-purified RSV F protein (a gift from Wyeth–Lederle–Praxis Biologicals, West Henrietta, New York) was diluted to a concentration of 250 ng/ml in carbonate/bicarbonate buffer, pH 9.6. A suspension (100 μl) containing 25 ng F protein was applied to wells of Immulon II 96-well plates (Nunc, Roskilde, Denmark). Blocking was accomplished overnight with 3% nonfat dry milk and 3% BSA. Blocking buffer (100 μl) containing 50 ng RhoA or Rac1, another Rho family GTPase (CalBiochem, La Jolla, California), and increasing concentrations of either $RhoA_{77-95}$ or $RhoA_{87-105}$ peptide (range, 100 ng/ml to 500 μg/ml) was added separately and incubated overnight at 4 °C. RhoA added without the peptide was used as a positive control. As a negative control, Rac1 protein and $RhoA_{87-105}$ were used instead of RhoA and $RhoA_{77-95}$, respectively. Monoclonal antibodies against RhoA or Rac1 (1:4,000 dilution; Santa Cruz Biotech, Santa Cruz, California) in blocking buffer were added after wells were washed with PBS and 0.1% Tween 20. After 1 h, plates were washed and goat antibody against mouse IgG conjugated to horseradish peroxidase (1:7,000 dilution) was added. After washing, the substrate 3,3',5,5'-tetramethylbenzidine (Sigma) was added and the absorbance was measured at 450 nm using a 'microtiter plate reader' (Dynatech, Chantilly, Virginia).

Treatment of $RhoA_{77-95}$ peptide in RSV-infected mice. Pathogen-free, 8-week-old BALB/c mice were obtained from Charles Rivers Laboratories (Raleigh, North Carolina) and were housed in a barrier facility. The mice were anesthetized and intranasally infected with 0.1 ml containing 1×10^6 PFU live RSV, as described[18]. Immediately before or 2 h or 4 d after RSV infection, 500 μg $RhoA_{77-95}$ peptide or PBS were given intranasally. Lungs were obtained from five mice from each group on day 4 after infection from RSV-infected mice treated with peptide or PBS immediately before or 2 h after

RSV infection. Five RSV-infected mice from each group were weighed for 12 d after infection. Illness was graded daily by an observer 'blinded' to treatment status of mice; clinical features of illness were scored as: 0, no apparent illness; 1, slightly ruffled fur; 2, ruffled fur, but active; 3, ruffled fur and inactive; 4, ruffled, inactive, hunched posture and gaunt; 5, dead.

RSV plaque assay from lung tissue. Four days after RSV infection, mice were killed by CO_2 narcosis and cervical dislocation. The lungs were removed, placed in EMEM with 10% FBS, and were quickly frozen in a bath of alcohol–dry ice. RSV titers in the lungs were measured by standard plaque assays[18] using HEp-2 monolayers that were 80% confluent. Lungs were thawed quickly and ground with a mortar and pestle. Serial 10-fold dilutions of lung supernatants were used to infect the monolayers in triplicate, and cultures were grown under 0.75% methylcellulose in EMEM with 10% FBS. Cells were formalin-fixed 5 d after being infected, and were stained with hematoxylin and eosin; plaques were counted using a dissecting microscope. Data are presented as the geometric mean $\log_{10}$ PFU per gram of lung tissue ± standard deviation (s.d.) at the dilution producing more than five plaques per well.

Blockade of PIV-3 infection in cell culture with peptides. Peptides $RhoA_{77\text{-}95}$ or $RhoA_{87\text{-}105}$ at concentrations of 50 μg/ml were incubated with 100 μl PIV-3 stock for 1 h on ice, then the suspension was added to HEp-2 cells in 12-well plates. PIV-3 plaques were viewed by phase contrast microscopy.

Acknowledgments

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Prophylaxis with Respiratory Syncytial Virus F–Specific Humanized Monoclonal Antibody Delays and Moderately Suppresses the Native Antibody Response but Does Not Impair Immunity to Late Rechallenge

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Respiratory syncytial virus (RSV) is the most significant viral cause of lower respiratory tract disease in infants and children. This study tested the hypothesis that a humanized murine monoclonal antibody (MAb) would protect against RSV infection in mice and have minimal suppressive effect upon the immune response because it is directed against a single epitope. A humanized murine MAb (RSHZ19) was tested for both prophylaxis and treatment of RSV infection in BALB/c mice and compared with a polyclonal product. Mice were rechallenged when passively administered antibody was undetectable (day 104). RSHZ19 reduced virus titer and protected against illness when used in prophylaxis and effected rapid virus clearance when used as treatment. Polyclonal antibody was also an effective prophylaxis but required 200 times the dose in total protein. Peak neutralizing antibody responses were delayed and somewhat suppressed in the prophylactically treated groups, but mice were protected against infection on rechallenge. Secondary antibody response to rechallenge in passively immunized mice was equal to that in untreated mice.

Respiratory syncytial virus (RSV) is universally recognized as the most important viral cause of lower respiratory tract disease in infancy and childhood. Accordingly, efforts have been focused on both prevention and treatment of this common illness. Prevention has been particularly targeted, in part because no therapeutic modality is consistently of significant benefit.

Prior studies in both animals and humans showed a role for passively administered antibody in the prevention of RSV infection, and both polyclonal and monoclonal antibody (MAb) preparations have been approved by the Food and Drug Administration for prophylaxis in infants at high risk for RSV infection [1–5]. A concern with the use of these products has been that passively administered antibody, although effective at preventing disease on primary challenge, may suppress antibody production, thereby diminishing immunity to rechallenge. In earlier studies, BALB/c mice given prophylactic immunoglobulin had no detectable neutralizing antibody responses 28 days after primary infection, and RSV titers in lungs upon rechallenge were inversely related to RSV titers after primary challenge [3].

The present study was designed to test the hypothesis that antibody suppression is clonal and determined by the specificity of the preparation used for treatment. Therefore, a MAb directed against a specific antigenic determinant of the fusion protein of RSV might have less suppressive effect upon the immune system than polyclonal serum because it leaves other antigenic determinants of the virus uncovered, allowing the immune system to develop antibodies against these other epitopes while still protecting against primary infection. Alternatively, the diminished antibody response may be due to antibody-mediated viral clearance, resulting in less viral antigenic stimulation of the immune system. If the latter theory is correct, then a MAb would cause as much suppression of the antibody response as polyclonal preparations.

Materials and Methods

Mice. Pathogen-free female BALB/c mice (8 weeks old; Charles River, Raleigh, NC) were cared for as previously described [6].

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All animals in these experiments were cared for in accordance with the Guide for the Care and Use of Laboratory Animals by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, and in accordance with Vanderbilt University guidelines.

S.B.D. is employed by SmithKline Beecham, the manufacturer of RSHZ19. No other authors have a commercial or other association that might pose a conflict of interest in objective evaluation of these data.

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Antibody. Humanized MAb of the IgG1κ isotype was supplied by SmithKline Beecham (King of Prussia, PA; RSHZ19 lot VJC-17622-187A). This antibody was initially produced in mice, and then the regions that determine murine complementarity were grafted onto a human IgG molecule [7]. The in vitro 60% plaque reduction neutralization titer of this antibody was 1 : 3325 at a concentration of 10 mg/mL. A dose of 5 mg/kg was administered intraperitoneally in 500 μL of PBS. In a pilot study, this dose was effective in preventing primary infection. The polyclonal antibody (Sandoglobulin lot 069; concentration 50 mg/mL) was provided by Val Hemming (Uniformed Services University of Health Sciences, Bethesda, MD). The 60% neutralization titer of this lot was 1:3265 at 10 mg/mL. It was administered at a dose of 1000 mg/kg, based again on the pilot study that showed protection at this dosage. A control humanized IgG1κ antibody directed against cytomegalovirus (CMV; CMHZ00, lot CS21035-155) was supplied by SmithKline Beecham.

Cells and virus. HEp-2 cells were maintained in Eagle MEM supplemented with glutamine, amphotericin, gentamicin, penicillin G, and 10% fetal bovine serum. The A2 strain of RSV was provided by Robert Chanock (NIH, Bethesda, MD). Working stocks of the virus were prepared as previously described [6].

Mouse infection. Anesthetized mice were infected nasally with 100 μL of undiluted stock as described previously [6]. A grading scale was used to assign numbers to a set of clinical features detected in mice with differing degrees of illness: 0, healthy; 1, barely ruffled fur; 2, ruffled fur but active; 3, ruffled fur and inactive; 4, ruffled fur, inactive, hunched, and gaunt; 5, dead. Illness scores were assigned by a blinded observer. Illness was also assessed by weighing the mice daily.

Plaque assays and neutralization tests. Two-day-old HEp-2 monolayers, 80% confluent in 12-well plates (Costar, Cambridge, MA), were used for plaque assays and neutralization tests. The assays were done as described [6] and have a lower limit of detection of 1.8 $\log_{10}$ pfu/g lung, 1.1 $\log_{10}$ pfu/nose, and 3.3 reciprocal $\log_2$ 60% neutralization titer.

ELISA for detection of passive MAb. We used an anti-idiotypic ELISA to measure serum concentrations of passively administered MAb as described [8]. The RSHZ19-specific anti-idiotypic MAb used as the capture antibody in this assay was generated by immunizing calves with the parent murine MAb (MAb19). RSHZ19 was quantitated with peroxidase-labeled anti-human IgG or with biotinylated anti-idiotypic MAb.

Histopathologic examination and scoring. Lung tissue was fixed in buffered formalin, processed routinely, and stained with hematoxylin-eosin. Alveolar inflammation (pneumonitis) was assessed as follows: 0, no infiltrate; 1, mixed generalized increase in interstitial mononuclear cells without widening of alveolar septa; 2, dense septal mononuclear infiltrates with thickening, with occasional foci of intra-alveolar cells; 3, significant alveolar consolidation in addition to interstitial inflammation, alveolar edema, or hemorrhage.

Experimental design. Mice in the prophylaxis group were given 5 mg/kg of RSHZ19 MAb preparation by intraperitoneal injection 18 h before intranasal challenge with 10^7 pfu of RSV. Controls for this group included 1 group that received an isotype-matched control MAb (directed against CMV protein [CMHZ00]) and 1 group that received 1 g/kg polyclonal immunoglobulin. Primary challenge mice that had received no prior treatment provided a positive control at the time of initial challenge and at rechallenge.

Mice in the treatment group were given RSHZ19 MAb 5 days after intranasal challenge with RSV. Control mice were given the control MAb CMHZ00 on the same day. All mice were weighed and assigned illness scores daily for 12 days after challenge.

Five mice from the prophylaxis group and 5 from each of its controls were euthanized on day 5, and plaque assays were done to assess virus titers in lung tissue. Mice in the treatment group and its controls were euthanized on day 6 for the same assays. Others were followed for progression of illness. Three mice in each group were euthanized on day 8, and their lungs were prepared for histopathologic examination.

One group of 5 mice received only the MAb; serial blood specimens were obtained biweekly to follow the disappearance of the MAb from sera and to ascertain that no traces of the passively administered antibody were present in the animals at the time of rechallenge (figure 1). For this reason, rechallenge was not done until day 104. Serum was drawn from all mice prior to rechallenge (to assess the primary antibody response) and 6 weeks after rechallenge (to assess the secondary antibody response). Mice were weighed and assigned illness scores, and 4 days after rechallenge 5 mice from each group were euthanized for plaque assays of the lungs and for histopathologic examination.

A second experiment, designed to examine the kinetics of neutralizing antibody response in animals given passive antibody, was done as follows: 8 mice were each given either RSHZ19 or the polyclonal immunoglobulin at the same doses as in the initial study. One control group of 7 mice was given control CMHZ00 MAb prophylaxis, and the other was given nothing (primary infection control). All mice were infected with 5×10^6 pfu of RSV 18 h after antibody administration. Mice in all groups were bled every 2 weeks, and their sera were tested for neutralizing antibodies. Reinfection was done on day 104, and secondary antibody titers were assayed at day 145.

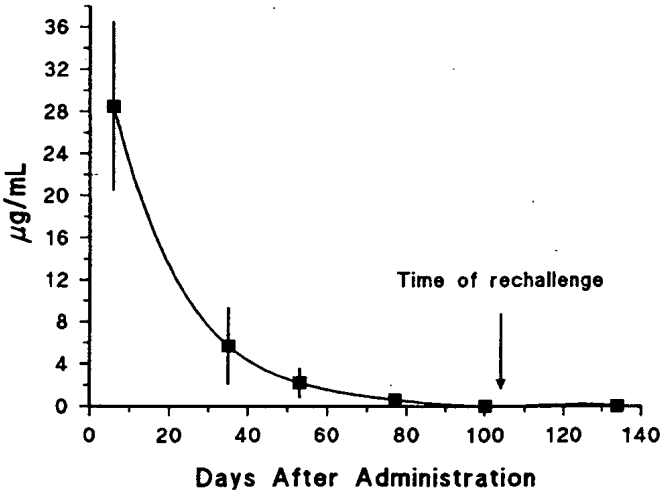


Figure 1. Time course of disappearance of passively administered RSHZ19 monoclonal antibody from sera of BALB/c mice. Each point represents geometric mean titer ± SD ($n = 5$).

Results

Primary Challenge

Viral replication. The titer of RSV in lungs of untreated mice 5 days after primary RSV infection was 6.0 $\log_{10}$ pfu/g. No virus was detected in lungs of mice given 5 mg/kg of RSHZ19 either as prophylaxis or as treatment. Virus was isolated from 3 of 5 mice in the group treated prophylactically with 1 g/kg polyclonal immunoglobulin with a geometric mean RSV titer of 3.1 $\log_{10}$ pfu/g (table 1).

Illness. On primary infection, animals given either RSHZ19 MAb or polyclonal immunoglobulin as prophylaxis had minimal weight loss. The MAb group was back to baseline weight within 7 days; weights in the polyclonal antibody group paralleled those of the MAb group. Weights in all other groups fell, reached a nadir at 7 days, and improved more gradually—returning to baseline weights by 12 days after infection. Illness scores were minimal in animals given anti-RSV MAb or polyclonal antibody prophylaxis. Scores in all other groups peaked at day 7 and returned to normal by day 12 (figure 2).

Pathology. Prophylaxis with either the RSV-specific MAb or polyclonal preparation was associated with reduction of interstitial infiltrates compared with the group treated with control CMHZ00 MAb. Of interest, the control CMHZ00 MAb given as prophylaxis diminished interstitial infiltrates relative to the control group treated with CMHZ00 on day 5. Mice given RSHZ19 MAb on day 5 as treatment also had a modest reduction in inflammation relative to CMHZ00-treated controls (data not shown).

Rechallenge

Viral replication. No virus was detectable in the lungs of mice in any treated group 4 days after rechallenge. The primary infection control mice, by contrast, had 7.2 $\log_{10}$ pfu/g of lung (table 1).

Illness. There was no significant illness or weight loss in any of the groups after rechallenge. Mice in the primary infection control group had severe illness and weight loss (mean loss of 6.4 g/mouse on day 7 after rechallenge; data not shown).

Pathology. Four days after rechallenge, all groups had peribronchiolar and periarteriolar lymphoid aggregates typically seen after rechallenge [9]; these were least prominent in mice given RSHZ19 MAb prophylactically. No neutrophilic infiltration or alveolar consolidation was observed in any group (data not shown).

Antibody Response

Primary challenge. Serologic analysis was performed in two separate experiments. In the first, plaque-reduction neutralizing activity was measured in serum prior to rechallenge on study day 104. Mice in the RSHZ19 MAb prophylaxis group had a geometric mean neutralization titer of 5.28 ± 1.9. Two of 9 had undetectable titers. In the polyclonal prophylaxis group, 1 of 10 had an undetectable titer. The mean neutralization titer was 5.4 ± 1.5 $\log_2$. Mice treated with RSHZ19 MAb 5 days after challenge had a significantly greater antibody response, with a geometric mean of 7.07 ± 0.7 $\log_2$ ($P = .02$). In the second study, we examined the kinetics of antibody response by evaluating a more complete time course after primary infection. At day 28, there was a significant difference in neutralizing antibody titers between the groups given RSHZ19 or polyclonal immunoglobulin prophylactically versus the control groups that received CMHZ00 or no treatment ($P = .01$). By 42 days after infection, however, the differences in neutralizing antibody titers among the groups were reduced and not statistically significant. Antibody titers in all groups continued to rise until day 56 after primary infection, then reached a plateau. Although all groups had sufficient antibody to protect from rechallenge on day 104, mice that were not given RSV-specific antibody prophylaxis reached a higher plateau ($P = .02$; figure 3).

Rechallenge. In the first study, antibody response was measured 4 weeks after secondary challenge on study day 132. Mice in the RSHZ19 MAb prophylaxis group had a geometric mean neutralization titer of 7.78 ± 2.13 $\log_2$. Titers in the polyclonal and control prophylaxis groups were 9.51 ± 1.13 $\log_2$ and 8.72 ± 0.23 $\log_2$, respectively. Mice treated with RSHZ19 MAb had a geometric mean neutralization titer of 8.98 ± 0.70 $\log_2$, and the CMHZ00-treated control group had a mean titer of 8.98 ± 1.1 $\log_2$. There were no statistically significant differences among these results. In the second study, the antibody response was measured on study day 145, 6 weeks after rechallenge. As in the first study, after rechallenge the neutralizing antibody responses achieved a similar titer in all groups despite having differing baselines (figure 3).

Table 1. Respiratory syncytial virus (RSV) titers in the lungs of mice treated with passive antibody.

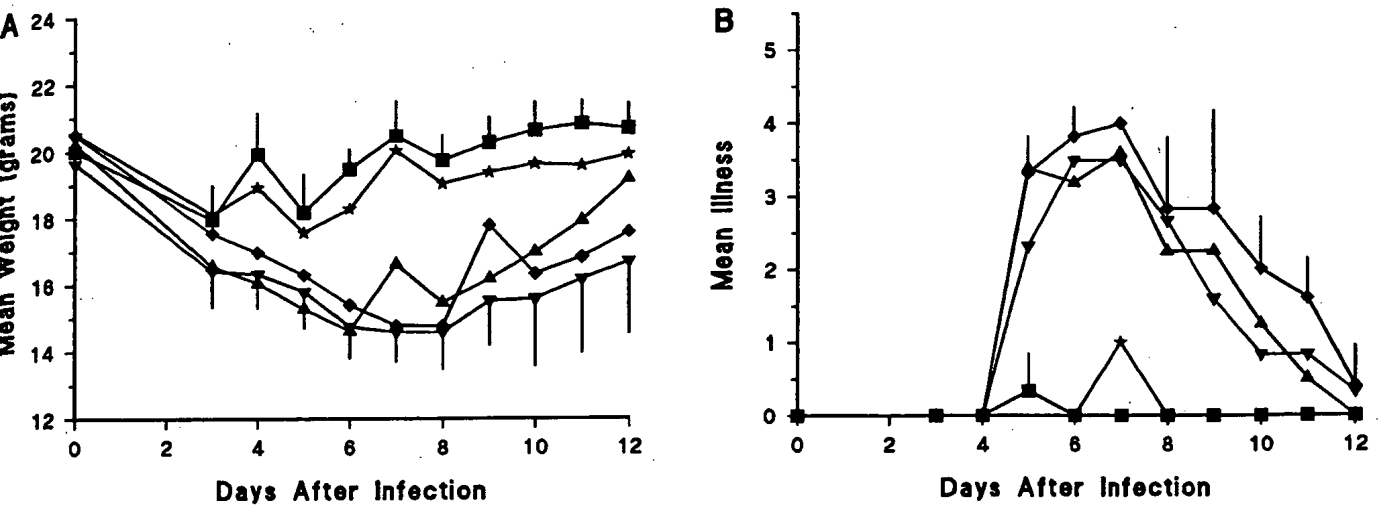
Treatment group	Treatment day	$\log_{10}$ pfu/g lung: Primary Infection Day 5	Primary Infection Day 6	Rechallenge Day 108
RSHZ19	−1	<1.8	ND	<1.8
CMHZ00	−1	5.4 ± 0.4	ND	<1.8
Polyclonal Ig	−1	3.1 ± 0.6	ND	<1.8
RSHZ19	5	ND	<1.8	<1.8
CMHZ00	5	ND	4.5 ± 0.6	<1.8
RSV PI control	ND	6.0 ± 0.2	3.7 ± 0.6	7.2 ± 0.2[a]

NOTE. Ig, immunoglobulin; ND, not done.

[a] This group of mice had RSV PI at day 104 as positive control for rechallenge virus stock. This is a typical RSV titer from a lung on day 4 after PI.

Discussion

The use of passively administered antibody for protection from and treatment of RSV infection decreases virus titer and



gure 2. Mean weight loss *(A)* and illness scores *(B)* after primary respriratory syncytial virus (RSV) infection in BALB/c mice. Each point presents mean ± SD of data from 5 animals. Mice treated prophylactically with RSHZ19 monoclonal antibody (■) or RSV-specific polyclonal mmunoglobulin (⋆) were protected from clinical signs of illness. Mice treated with CMHZ00 prophylactically (▼) and groups treated on day 5 th RSHZ19 (▲) or CMHZ00 (◆) had typical patterns of illness and weight loss.

revents severe illness in several model systems. Its suppressive fects upon the humoral response to infection have been a use for concern about widespread use of this therapy because the possibility of multiple exposures to RSV in the course a single RSV epidemic. The phenomenon of "antibody feedck" has been observed with other passively administered anodies [10, 11]. Studies on the specificity of the phenomenon ve shown that it is determinant specific; mice will have supession of antibody response to one antigen while retaining rmal humoral response to another antigen presented on the me cell [12]. These data provided a basis for the hypothesis at if the humoral response was suppressed by passively adnistered MAb, it would be suppressed only to a single anenic determinant on RSV.

The present study shows that a relatively low dose of pasely administered RSV-specific humanized MAb can reduce rus titers to below detectable limits after primary RSV infecn in BALB/c mice. Mice given the MAb prophylactically o had decreased illness compared with untreated controls or th control mice given an isotype-matched CMV-specific conl MAb. In mice given RSHZ19 as treatment for an estabhed infection, virus was rapidly cleared, but clinical illness d weight loss were not affected. This is similar to the clinical perience in which administration of an RSV-specific humand MAb to hospitalized RSV-infected infants reduced virus ers but had no measurable clinical effect [13]. The polyclonal eparation also reduced virus titers after primary challenge. o of 5 mice had no detectable virus; in the other 3, virus ers were reduced ~1000-fold. However, it required more an-

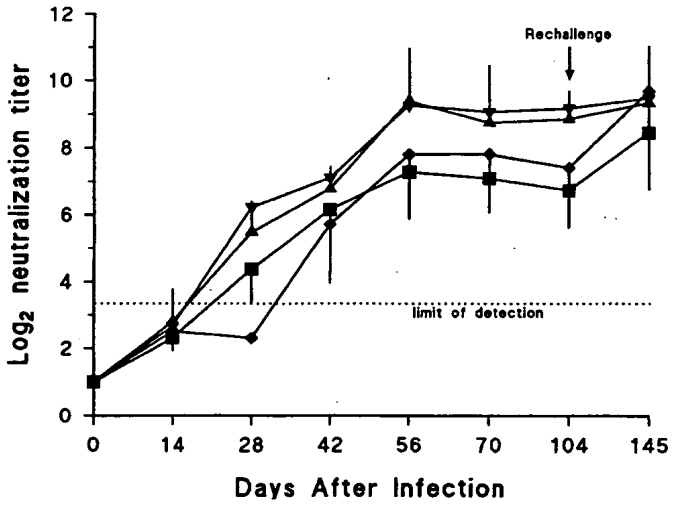


Figure 3. Kinetics of antibody production in mice given prophylactic monoclonal antibodies (MAb): time course of $\log_2$ 60% neutralization titers of mice given RSHZ19 MAb (■), control CMHZ00 MAb (▲), respiratory syncytial virus–specific polyclonal immunoglobulin (◆), or nothing (▼) and then infected with RSV intranasally. Each data point represents geometric mean ± SD of 6–8 mice. At day 104 mice were rechallenged with live RSV, and secondary neutralizing antibody responses were assayed at day 145. Limit of detection for this assay is ~3.3 $\log_2$. Mice with undetectable neutralization titers were assigned a value one-half the lower limit of detection for statistical calculations.

tibody to protect mice with the polyclonal preparation; they were given 200 times the amount of total protein to afford similar protection.

In a prior passive polyclonal antibody prophylaxis study done by our group, neutralizing antibody assays 28 days after primary infection showed no detectable neutralizing antibody. [3] These mice also proved to be susceptible to infection after rechallenge on day 28. Others have also shown a lack of neutralizing antibody responses in animals given passive antibody prophylactically, although susceptibility to rechallenge has varied [14, 15].

In the present study, mice were not rechallenged until day 104, when passively administered antibody was completely cleared from serum (figure 1). Sera from mice treated prophylactically with RSV-specific MAb or polyclonal immunoglobulin drawn at day 104 had detectable neutralizing activity, but titers were lower than those produced in control groups. The data in figure 3 provide further confirmation that passive antibody treatment alters both the kinetics and the magnitude of antibody response to RSV infection but does not fully suppress it, thus providing an explanation for why antibody was not detected in earlier studies in which mice were rechallenged at day 28. The phenomenon of delayed antibody response due to passively administered immunoglobulin has been previously reported in animal [16] and human [17] studies. This is probably related to the early reduction of antigen load suggested by the higher neutralizing titers seen in mice that received RSHZ19 just 5 days later.

In the present study, neutralizing antibody responses, although present, were still diminished. Nevertheless, mice in both the RSHZ19 MAb and polyclonal immunoglobulin groups were resistant to infection on rechallenge. Moreover, some of the mice in each group, despite undetectable levels of serum antibody, were completely protected on rechallenge. The fact that animals given the RSV-specific MAb prophylactically were completely protected against rechallenge even though they had no recoverable virus on primary challenge shows that an effective immune response was generated despite limited viral replication. Others showed that cotton rats prophylactically treated with a similar RSV F-specific humanized Mab were also protected from rechallenge [18]. In addition, protection from reinfection despite weak antibody responses has been noted in animal models of influenza [14, 15]. In summary, a humanized RSV-specific MAb was effective in preventing illness and reducing virus titers below detectable limits after primary RSV challenge. Mice were also protected from secondary infection despite treatment during primary infection with either RSV-specific MAb or polyclonal immunoglobulin. Passive antibody given prophylactically delayed the development of serum neutralizing antibody response but caused only a modest decrease in the magnitude of the ultimate titer. Suppression of the humoral immune response did not differ greatly between the monoclonal and polyclonal preparations, suggesting that the total antigen load was a major determinant of the kinetics of primary antibody response. There was neither clinical nor histopathologic evidence of enhanced illness or aberrant immune responses in mice receiving passive antibody.

Prophylactic or therapeutic treatment with RSV-specific humanized IgG MAb did not increase susceptibility to late reinfection, and secondary neutralizing antibody responses were preserved. These results suggest that RSV-specific antibody prophylaxis for primary RSV infection should not compromise the ability of treated infants to mount an effective immune response to subsequent RSV infection.

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EDIATRIC RESEARCH

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Immunoglobulin Administration and Ribavirin Therapy: Efficacy in Respiratory Syncytial Virus Infection of the Cotton Rat

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ABSTRACT. We studied the effects of combined administration of human immunoglobulin (IVIG) and ribavirin aerosol on respiratory syncytial virus (RSV) infection in cotton rats (*Sigmodon hispidus*). Cotton rats assigned to receive combined therapy were administered Gamimune, a preparation of purified IVIG with a high titer of anti-RSV neutralizing activity, intraperitoneally 24 h prior to intranasal RSV challenge and then treated with ribavirin aerosol 3 days after challenge. Lung viral titers from these cotton rats (geometric mean titers [GMT] $\log_{10} = 0.15 \pm 0.5$) were lower than titers from untreated animals (GMT, $\log_{10} = 3.7 \pm 0.6$) and animals treated with either IVIG alone (GMT, $\log_{10} = 1.8 \pm 0.9$) or ribavirin alone (GMT, $\log_{10} = 1.9 \pm 1.1$). Only one of 12 cotton rats treated with both IVIG and ribavirin had a demonstrable titer of virus after RSV challenge. When IVIG administration was delayed until day 3 after virus challenge, lung viral titers were still lowest in animals receiving both IVIG and ribavirin. In comparison, there was no additive antiviral effect between IVIG and ribavirin against RSV infections of HEp-2 cells *in vitro*. Pathologic changes on histologic examination of pulmonary tissues from animals challenged with RSV were least prominent in animals treated with both IVIG and ribavirin. Despite the apparent absence of *in vitro* additive antiviral effect, combined use of IVIG and ribavirin was more efficacious against RSV infection in the cotton rat than use of either agent alone. (*Pediatr Res* 21: 270–274, 1987)

Abbreviations

RSV, respiratory syncytial virus
IVIG, human immunoglobulin
$TCID_{50}$, tissue culture infections doses
MEM, minimal essential medium
SFU, syncytia forming units
PBS, phosphate-buffered saline
GMT, geometric mean titers

RSV is the most important viral cause of lower respiratory ct disease in infants and young children (1) and is associated th significant morbidity in children with underlying cardio-lmonary disease (2). Until an effective vaccine against RSV n be developed, effective protection of high-risk infants against serious RSV disease may require treatment with combinations of currently available therapeutic agents.

Previous studies have shown that parenteral inoculation of IVIG containing a high titer of anti-RSV neutralizing activity can provide significant protection in the cotton rat against RSV infection, (3) and significantly reduce the amount of virus shed from the airways of RSV infected owl monkeys (4). Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a synthetic nucleoside that possesses inhibitory activity against RSV *in vitro*, has been shown to reduce the amount of RSV in lung tissue of experimentally infected cotton rats (6) and appears to exert a beneficial effect on the course of human RSV infection (7–9). The present study evaluated the combined effect of IVIG and ribavirin on the quantity of RSV contained in lungs of experimentally infected cotton rats and the effect of this combination on histopathologic evidence of infection.

METHODS

Animals. Cotton rats (*Sigmodon hispidus*) used in these studies were derived from two pairs of animals obtained from the Small Animal Section, Veterinary Resources Branch, Division of Research Services, National Institutes of Health. Test animals were 2–3 wk old at the start of each experiment. All animals were maintained in cages with barrier filters and fed food and water *ad libitum*.

Experimental design. Animals were randomly assigned to receive IVIG alone (group 1), ribavirin alone (group 2), IVIG + ribavirin (group 3), or no treatment (group 4). Animals designated to receive prophylaxis with IVIG (groups 1 and 3) were lightly anesthetized with ether and 0.5 ml of IVIG was administered intraperitoneally. Twenty-four h after administration of antisera, the cotton rats from all four groups were anesthetized with ether, bled from the retroorbital venous plexus, and challenged intranasally with 10^4 median $TCID_{50}$ of RSV. Three days after virus challenge animals in groups 2 and 3 were treated with ribavirin delivered continuously by small particle aerosol for 18 h. On day 4 after challenge, blood specimens were obtained, and lungs were harvested from each animal. Cotton rats that were treated with ribavirin were sacrificed 3 h after aerosol delivery was completed. The left lingular region of each lung was placed in formalin, embedded in paraffin, sectioned (5 μ), and stained and hematoxylin and eosin. These sections were rank ordered blindly based on overall assessment of histopathology. The remaining lung tissue was homogenized and assessed for virus titer. Otherwise identical experiments were performed in which IVIG administration was delayed until day 3 after RSV intranasal challenge. In order to rule out *in vitro* neutralization of RSV by IVIG or ribavirin that might be present in lung tissue at the time that the lungs were harvested, parallel experiments were performed in which cotton rats were assigned to groups 1–4 as noted above, but the animals were not challenged with RSV. Instead,

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lungs from these animals were harvested, homogenized, and serially diluted 2-fold in 96-well microtiter plates, and 35 $TCID_{50}$ of RSV were then added to each well. After plates were maintained 2 h at room temperature, 0.05 ml of a suspension of HEp-2 cells (10^4 cells/ml) were added to each well. At the end of a 7-day incubation period at 37° C in a humidified incubator with 5% CO_2, the extent of inhibition of the challenge virus by lung homogenates from each group was determined.

Virus. The Long strain of RSV, propagated in HEp-2 cells, was used in all experiments.

Virus assay. Lungs were homogenized in 1 ml of Earle's MEM (Gibco, Grand Island, NY) supplemented with 5% fetal calf serum (Gibco) and assayed immediately for the presence of RSV. Briefly, half log_{10} dilutions of lung specimens were added in duplicate to monolayers of HEp-2 cells (Flow Laboratories, McLean, VA) grown in 16-mm diameter wells. After a 60-min adsorption period at 37° C in a 5% CO_2 atmosphere, Earle's MEM containing 5% fetal calf serum was added, and the plates were placed in a 37° C (5% CO_2) incubator for 5 days. At that time the monolayers were fixed and stained with 0.5% aqueous crystal violet in 70% methanol and SFU counted. Viral titers were expressed as log_{10} SFU/lung.

Immune sera. Gamimune (Lot no. 45N18; Cutter Biologicals, Berkeley, CA), a preparation of purified human IgG with a RSV neutralizing antibody titer of 1:3162, was used in all experiments.

Ribavirin administration. Ribavirin (Viratek, ICN Pharmaceuticals, Costa Mesa, CA) was prepared as a 15 mg/ml solution and delivered by small particle aerosol generated by a Collison nebulizer over 18 h, as previously described (10, 11).

Antibody assay. Anti-RSV antibody in cotton rat sera was measured by modification of a microneutralization assay previously described (12). Briefly, all sera were heated at 56° C for 30 min prior to testing. Duplicate 0.025-ml volumes of 2-fold serum dilutions of the heat-inactivated sera were then prepared in 96-well plates and mixed with equal volumes of a RSV pool containing approximately 100 $TCID_{50}$ per 0.025 ml. After 2 h at room temperature 0.05 ml of a suspension of HEp-2 cells (10^4 cells/ml) was added to each well and the plates were incubated at 37° C in a humidified incubator with 5% CO_2. The test was read each day for 5 days. Titers were defined as the reciprocal of the last dilution of antisera that reduced SFU ≥50% as compared to the mean number of SFU in virus control wells.

Antiviral effect of IVIG and ribavirin in vitro. The effects of IVIG and ribavirin alone and in combination on RSV infection in tissue culture were studied. Gamimune was diluted with PBS (pH 7.2) to a starting concentration providing an RSV neutralizing titer of 1:8. Ribavirin was similarly diluted to a starting concentration of 40 μg/ml. Two-fold dilutions using MEM supplemented with 5% fetal calf serum were made with each agent alone and with respect to each other in 96-well microtiter plates. One hundred $TCID_{50}$ of RSV were added to each well. After incubation at room temperature for 2 h, HEp-2 cells were added to each well and plates were incubated at 37° C (5% CO_2) for 5 days at which time each well was observed for cytopathic effect. Titer was defined as the reciprocal of the last dilution of antiserum (log_2), ribavirin, or a combination of the two which reduced SFU ≥50% in comparison to virus plus medium controls.

Statistics. GMT, SD, χ^2, and F tests were performed as described by Sokal and Rohlf (13). Bonferroni χ^2 tests, *t* tests, or Wilcoxon rank sum tests were performed on paired groups (14).

RESULTS

Cotton rat sera from animals which received IVIG 24 h prior to virus challenge (groups 1 and 3) demonstrated an RSV neutralizing antibody GMT (log_2) of 4.9 and 5.0 ± 1.4, respectively, on the day of challenge (day 0) and a GMT of 3.8 and 3.5 ± 1.0, respectively, on the day of sacrifice (day 4). Sera from untreated cotton rats or animals that received ribavirin alone did not demonstrate detectable RSV neutralizing activity (Table 1).

Virus titers and the percentage of cotton rats in each group which had virus isolated from lung tissue are shown in Table 2. Initial analysis using the χ^2 test indicated that there were significant differences between groups ($\chi^2 = 27.6$, $p < 0.001$). However, among groups treated with either IVIG or ribavirin alone, the number of animals with detectable virus in lung tissue did not differ significantly from the untreated group. In contrast, the group of cotton rats treated with both IVIG and ribavirin had significantly fewer animals that had detectable lung virus titers when compared to groups of untreated animals or to cotton rats treated with either agent alone ($p < 0.001$, $p < 0.005$ using Bonferroni χ^2).

Differences in lung virus titers were observed between groups of animals ($F = 36.57$, $p < 0.001$) (Table 2). Animals receiving ribavirin or IVIG alone had significantly lower titers of RSV in lung tissue than untreated animals ($p < 0.003$, Bonferroni two-tailed *t* test) but were not significantly different from each other. Animals that received both agents, however, had significantly lower virus titers than untreated animals ($p < 0.001$) or animals receiving either IVIG or ribavirin alone ($p < 0.005$). When IVIG administration was delayed until day 3 after RSV challenge, the group of cotton rats treated with both IVIG and ribavirin still had fewer animals with detectable lung virus. In addition, lung virus titers were lowest in animals that received both agents. However, these results were not statistically significant when compared to results obtained from animals which received either ribavirin or IVIG alone (Table 3).

Normal cotton rat lung histology is shown in Figure 1. Histopathologic examination of lung tissue from untreated animals and from those receiving ribavirin alone revealed peribronchial

Table 1. *Neutralization titers to RSV in microneutralization assays of sera from cotton rats administered IVIG, ribavirin, IVIG + ribavirin, or no treatment*

Group	Treatment (*n*)	Neutralization titer (log_2) (GMT ± SD)* Day 0	Day 4
1	IVIG (15)	4.9 ± 1.0	3.8 ± 1.0
2	Ribavirin (15)	<2	<2
3	Ribavirin + IVIG (14)	5.0 ± 1.4	3.5 ± 1.0
4	None (16)	<2	<2

* Titer = reciprocal of the last dilution (log_2) which produced a 50% or greater reduction in syncytia in comparison to virus + media controls.

Table 2. *Isolation and titers of RSV from lung homogenates of cotton rats administered IVIG, ribavirin, or IVIG + ribavirin*

Group	Treatment (*n*)	No. of cotton rats with detectable virus (%)*	Titer of virus in lung homogenates (log_{10} SFU/lung) (GMT ± SD)†
1	IVIG (11)	9 (80)	1.8 ± 0.9‡
2	Ribavirin (12)	10 (83)	1.9 ± 1.1‡
3	IVIG + ribavirin (12)	1 (8)§	0.14 ± 0.5‖
4	None	12 (100)	3.7 ± 0.6

* $\chi^2 = 27.6$, $p < 0.001$.

† $F = 35.57$, $p < 0.001$. Lung virus titers less than 1 log_{10} were considered to be 0 log_{10} for purposes of statistical analysis.

‡ $p < 0.003$ in comparison to no treatment group (Bonferroni two-tailed *t* test).

§ $p < 0.001$ in comparison to no treatment group, $p < 0.005$ in comparison to groups treated with ribavirin alone or IVIG alone (Bonferroni χ^2).

‖ $p < 0.001$ in comparison to GMT of no treatment group, $p < 0.005$ in comparison to GMT of group treated with ribavirin alone or IVIG alone (Bonferroni two-tailed *t* test).

peribronchiolar inflammatory infiltrates with ballooning, ping, and exfoliation of eosinophilic epithelial cells. Little ence in the histopathology of the lungs in these two groups bserved (Figs. 2 and 3). IVIG administration appeared to ct against tissue damage, as evidenced by preservation of architecture and reduction in pulmonary inflammatory ates which were most prominent in animals receiving both and ribavirin (Fig. 4). Lungs from cotton rats that received prior to RSV challenge followed by ribavirin therapy on demonstrated histology which was difficult to distinguish that of uninfected lungs.

rallel experiments performed in animals that were not chal- d with RSV revealed that *in vitro* neutralization of added (35 $TCID_{50}$) by homogenates of lungs from treatment ps was no different than that produced by homogenates of from untreated animals (Table 4). These data suggest that ction of viral titers observed in treatment groups was not o *in vitro* inactivation of virus by residual IVIG or ribavirin rvested lungs, but rather represented an *in vivo* effect.

e performed *in vitro* tests to look for synergistic or additive ts when ribavirin and IVIG were combined. Gamimune was diluted to a concentration providing an RSV neutralizing titer of 1:8. (A 1:16 dilution of the preparation did not inactivate virus.) Ribavirin alone inactivated RSV at a concentration of 20 μg/ml but 10 μg/ml did not limit viral replication in HEp-2 cells. However, the combination of a 1:16 dilution of the above IVIG preparation and 10 μg/ml of ribavirin prevented viral cytopathic effect in HEp-2 cells. Further dilution of either agent in combination with a fixed concentration of the other failed to inactivate the virus. Therefore *in vitro* additive effect of the two agents in combination was minimal.

le 3. *Isolation and titers of RSV from lung homogenates of cotton rats treated on day 3 with IVIG, ribavirin, or IVIG + ribavirin*

oup	Treatment (*n*)	No. of cotton rats with detectable virus (%)*	Titer of virus in lung homogenates (log_{10} SFU/lung) (GMT ± SD)†
1	IVIG (8)	6 (75)	1.5 ± 0.9‡
2	Ribavirin (7)	6 (86)	2.1 ± 1.1
3	IVIG + Ribavirin (8)	3 (38)	0.75 ± 1.0§
4	None (8)	8 (100)	3.4 ± 0.7

$\chi^2 = 6.42$, $p < 0.1$.

$F = 11.01$, $p < 0.001$. Lung virus titers less than 1 log_{10} were idered to be 0 log_{10} for purposes of statistical analysis.

$p < 0.05$ in comparison to no treatment group (Bonferroni two- d Wilcoxon rank sum test).

$p < 0.05$ in comparison to no treatment group, $p > 0.1$ to groups ted with IVIG or Ribavirin alone (Bonferroni two-tailed Wilcoxon sum test).

DISCUSSION

In 1973, Parrott *et al.* (15) observed that infants less than 2 months of age who had high titers of maternally derived anti-RSV serum antibodies experienced bronchiolitis less frequently than older infants who had lower levels of serum antibodies. Subsequently, Glezen *et al.* (12) and others (17) demonstrated that RSV disease occurs less often and is less severe in infants with high levels of passively acquired maternal RSV antibodies. Experiments in the cotton rat indicated that a high titer of passively administered serum RSV neutralizing antibody protected the lungs against infection with RSV and that the levels of serum antibody correlated with resistance to infection (16). In subsequent experiments passive inoculation with IVIG provided significant protection against RSV lung infection in the cotton rat (3) and significantly reduced amounts of virus shed from the noses and airways of RSV-infected owl monkeys (4).

Although purified IVIG preparations with high titers of anti-RSV neutralizing activity might be useful in protecting high-risk infants, some investigators have reported considerable overlap in the level of maternally derived serum neutralizing antibodies among infants who develop severe RSV disease and those who do not (17). This finding suggests that administration of IVIG containing RSV neutralizing antibody may not provide uniform protection for high-risk infants and combination therapy with another agent active against RSV might prove to be more effective.

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a synthetic nucleoside that is a structural analog of guanosine, has shown promising activity against RSV *in vitro* and *in vivo*. This drug when delivered by aerosol reduces the amount of RSV in lung tissues of experimentally infected cotton rats (6) and appears to ameliorate human infection with particularly striking improvement in arterial oxygen pressures in infected infants (9).

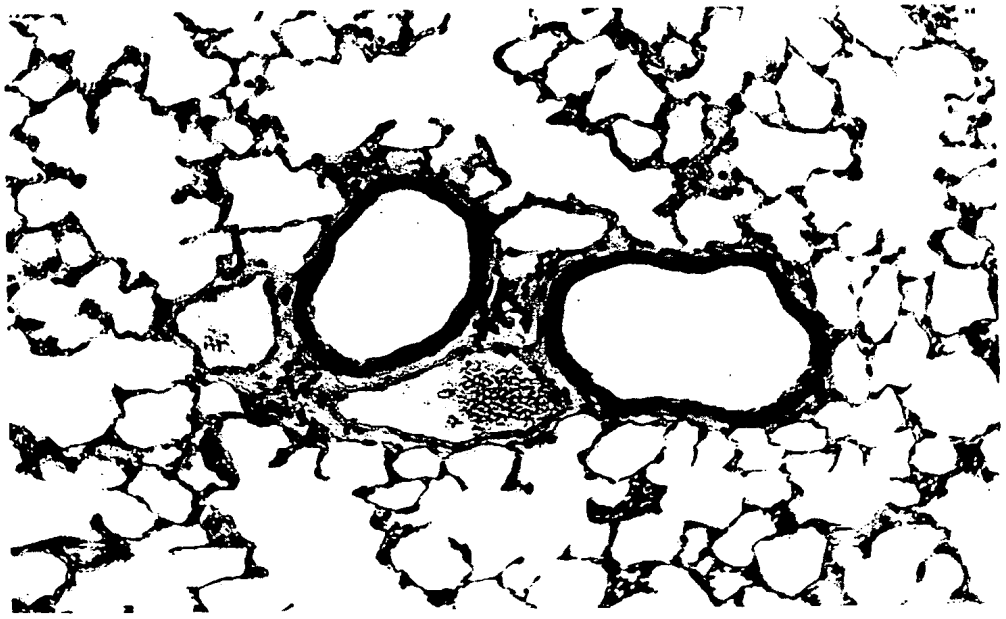


Fig. 1. Normal cotton rat lung. This and all subsequent sections were prepared by fixing lung tissue in formalin and staining paraffin-embedded tions with hematoxylin and eosin. Magnification, ×100.

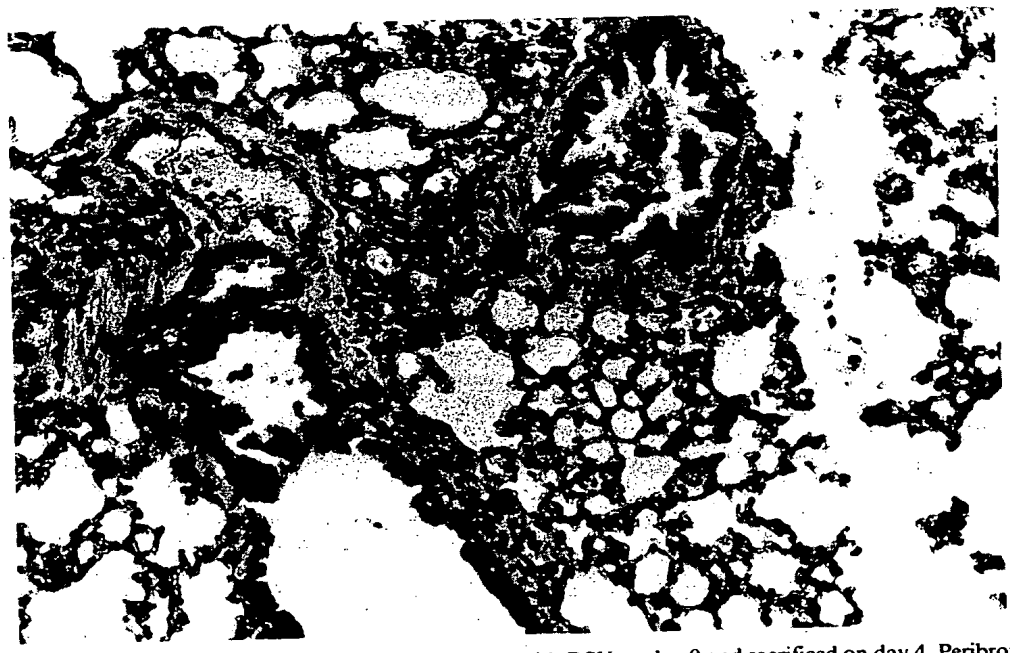


Fig. 2. Control cotton rat lung. The animal was challenged intranasally with RSV on day 0 and sacrificed on day 4. Peribronchiolar inflammation is present and bronchiolar epithelial cells show ballooning, clumping, and exfoliation. Magnification, ×100.

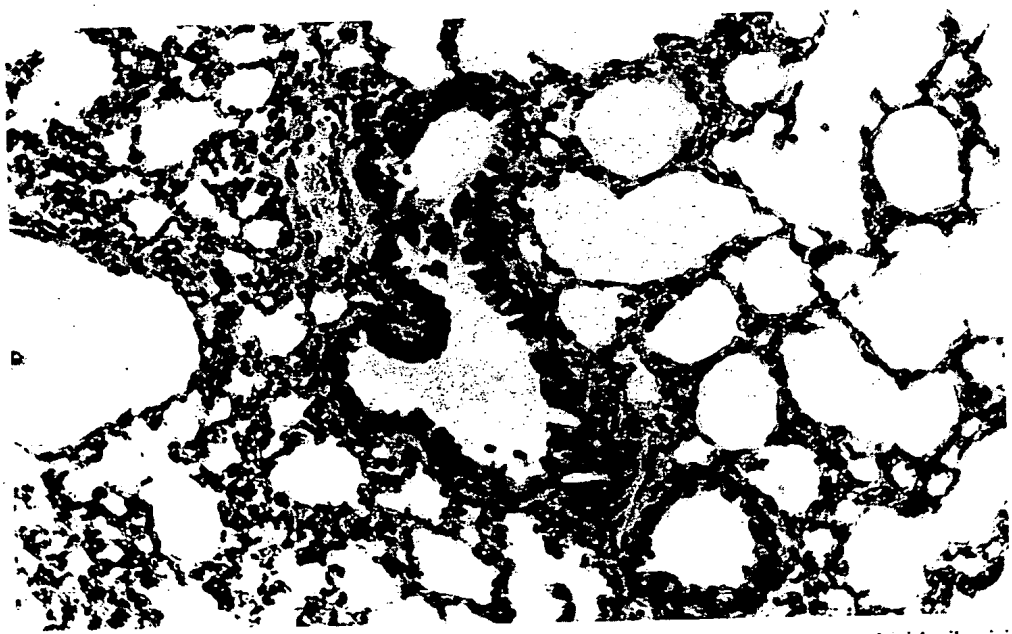
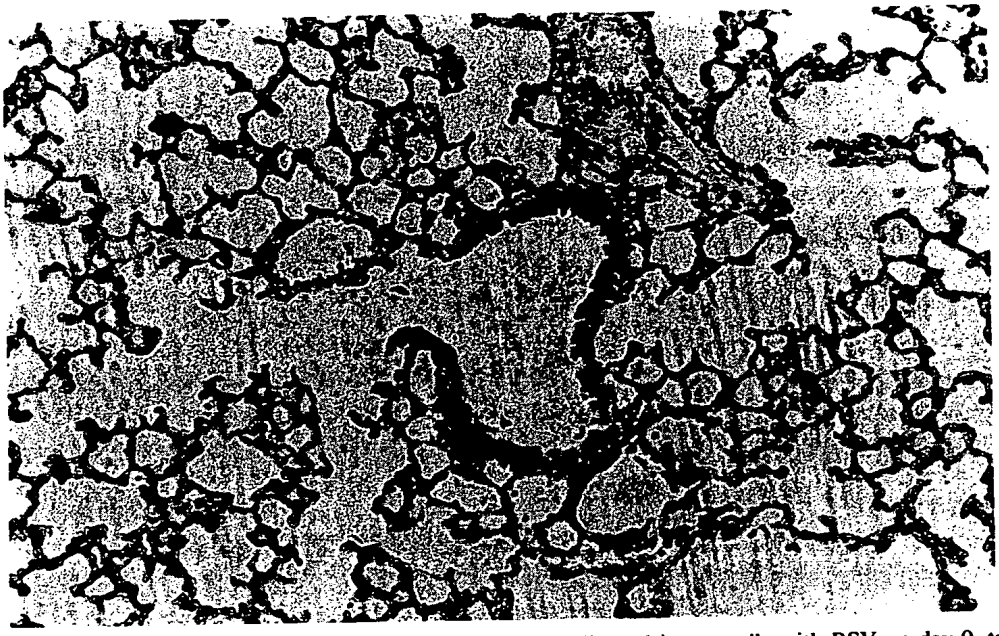


Fig. 3. Lung from ribavirin-treated cotton rat. Animal was challenged intranasally with RSV day 0, treated with ribavirin day 3, and sacrificed day 4. Bronchiolar epithelial cell stratification, clumping and cell loss is similar to that seen in control lungs. Magnification, ×100.

However, subjective improvement is often subtle (8), and in one study the proportion of patients shedding RSV from their nasal secretions during the first 3 days of ribavirin therapy did not differ from a group receiving placebo (9). Combination of ribavirin with another agent might provide improved protection against RSV infection.

In the current study we investigated whether administration of IVIG in combination with ribavirin therapy by small particle aerosol provided advantages over either agent alone against RSV infection in the cotton rat. GMT of anti-RSV specific neutralizing antibody in serum of cotton rats who received IVIG were comparable to passively acquired antibody titers of infants protected against severe RSV disease (12). Animals who received IVIG or ribavirin alone showed a significant reduction in amounts of virus isolated from lung homogenates, but numbers of animals with detectable virus did not differ from untreated animals. In comparison, animals treated with both IVIG and ribavirin had significantly lower lung viral titers than untreated animals or animals treated with either agent alone, and only one of 12 (8%) cotton rats who received IVIG prophylaxis followed by ribavirin therapy on day 3 had detectable virus in lung tissue at the time of sacrifice. This latter regimen appeared to preserve normal lung histology. These results could not be explained by *in vitro* inactivation of virus, nor was an *in vitro* additive effect demonstrable between IVIG and ribavirin. We obtained similar results when IVIG administration was combined with ribavirin therapy on day 3, although results were less striking; three of eight (38%) cotton rats who received combination therapy had



ig. 4. Lung from IVIG and ribavirin-treated cotton rat. The animal was challenged intranasally with RSV on day 0, treated with IVIG and ribavirin on day 3, and sacrificed on day 4. Bronchiolar architecture is relatively preserved with mild epithelial stratification and cell loss. gnification, ×100.

able 4. *Neutralization of RSV (35 TCID^{50}) in vitro by lung omogenates from cotton rats administered IVIG, ribavirin, or IVIG + ribavirin*

Group	Treatment (*n*)	Neutralization titer (log^2) (GMT ± SD)*†
1	IVIG (4)	1.25 ± 0.5
2	Ribavirin (4)	1.25 ± 0.5
3	Ribavirin + IVIG (4)	1.5 ± 0.6
4	None (4)	1.5 ± 0.6

Titer = reciprocal of the last dilution (log_2) which produced a 50% greater reduction in syncytia in comparison to virus + media controls.
i $F = 1.3$, $p > 0.25$.

tectable lung virus at the time of sacrifice and mild pulmonary topathology was noted.

A possible interpretation of the above findings is that human ti-RSV IgG antibody combined with cellular or humoral immune responses of the cotton rat to limit RSV replication and at ribavirin therapy following infection neutralized the remaining low titer of virus. This interpretation is supported by the fact at animals that received IVIG prior to RSV infection demonated less histopathology than animals that did not, and animals at received both ribavirin and IVIG had the greatest preservan of normal lung appearance. Further studies are indicated to termine how IVIG and ribavirin interact with local antibody d cellular elements (*e.g.* macrophages, cytotoxic T cells) to nit RSV infection.

The success of IVIG combined with ribavirin in eradicating SV and protecting the lungs of cotton rats may have important plications for successful intervention against RSV infection in gh-risk infants. A potential strategy would be to administer IG prophylaxis to high-risk infants at a time when RSV is entified in the community in the hope of preventing RSV fection. Infants who develop RSV illness despite prophylaxis uld then be treated with ribavirin. Alternatively, IVIG and avirin could be administered in combination during estabhed RSV infections of high-risk infants. Experience with comnation therapy in the cotton rat suggests that such strategies ay prove more effective than those that rely on either IVIG or avirin alone. Clinical trials are indicated to determine the best proach.

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RhoA Interacts with the Fusion Glycoprotein of Respiratory Syncytial Virus and Facilitates Virus-Induced Syncytium Formation

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The fusion glycoprotein (F) of respiratory syncytial virus (RSV), which mediates membrane fusion and virus entry, was shown to bind RhoA, a small GTPase, in yeast two-hybrid interaction studies. The interaction was confirmed in vivo by mammalian two-hybrid assay and in RSV-infected HEp-2 cells by coimmunoprecipitation. Furthermore, the interaction of F with RhoA was confirmed in vitro by enzyme-linked immunosorbent assay and biomolecular interaction analysis. Yeast two-hybrid interaction studies with various deletion mutants of F and with RhoA indicate that the key binding domains of these proteins are contained within, or overlap, amino acids 146 to 155 and 67 to 110, respectively. The biological significance of this interaction was studied in RSV-infected HEp-2 cells that were stably transfected to overexpress RhoA. There was a positive correlation between RhoA expression and RSV syncytium formation, indicating that RhoA can facilitate RSV-induced syncytium formation.

Human respiratory syncytial virus (RSV) belongs to the *Pneumovirus* genus of the *Paramyxoviridae* family. RSV is the major cause of acute lower respiratory tract illness in infants and young children (reviewed in reference 9). RSV isolates have been classified into two antigenic subgroups (A and B) on the basis of differences in reactivity with panels of monoclonal antibodies to attachment (G) protein (1, 34). The RSV envelope contains two major glycoproteins, the G and fusion (F) glycoproteins. The G glycoprotein is thought to mediate virus attachment (29), but the cell receptor has not been defined. The F glycoprotein promotes fusion of the viral and cellular membranes with subsequent transfer of viral genetic material into the cell. The F glycoprotein also promotes fusion of infected cell membrane with adjacent cell membrane, leading to the formation of syncytia. A third protein, the small hydrophobic (SH) protein, is also present in the envelope, but its function is unknown.

The F glycoprotein is synthesized as an inactive precursor, F0, which is cotranslationally modified by the addition of N-linked glycosylation in the endoplasmic reticulum. The F0 precursor is thought to assemble as a homooligomer into a tetramer (8). The F0 precursor is cleaved by cellular trypsin-like endoproteases into two disulfide-linked subunits, F1 and F2, before reaching the cell surface. The RSV F protein is structurally similar to the F proteins of other paramyxoviruses (6).

Three virus-encoded proteins, the nucleocapsid (N) protein, the phosphoprotein (P), and the RNA polymerase (L), are associated with the nucleocapsid to form a transcribing ribonucleoprotein (RNP) complex. RSV uses an additional protein expressed from the M2 gene open reading frame 1 as a transcription elongation factor (7). Previous studies indicate that the RNP complex requires cellular actin and possibly other proteins for RSV transcription (5, 19, 24). Similar involvement of cytoskeleton proteins in transcription has been observed in several other paramyxoviruses, namely, Sendai virus, measles virus, and parainfluenza virus type 3 (12, 32, 33). The interaction of RNP and the polymeric form of actin results in the alteration of structure of RNP from a loosely coiled to a moderately condensed form which appears to be favorable for transcription (12).

In addition, many enveloped viruses utilize cellular actin during the process of budding and maturation of virus particles released from the infected cells (4, 11, 44, 50). Furthermore, actin microfilaments have recently been shown to be involved in the spread of vaccinia virus between cells (10). Therefore, many enveloped viruses in general may use a common strategy for their transcription, morphogenesis, and cell-to-cell spread by utilizing cellular cytoskeletal components.

RhoA, a small GTPase of the Ras superfamily, has been shown to control a plethora of biological functions, including actin reorganization, gene expression, cell morphology, cell motility, and cell proliferation (35). RhoA is a common target for bacterial toxins and is of major importance for the entry of bacteria such as *Shigella* and *Salmonella* spp. into mammalian host cells (26, 51). It is also important in cell transformation by polyomaviruses (48). Further, it has been shown that adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases (30).

RhoA cycles between two states, i.e., an inactive, GDP-bound form and an active, GTP-bound form. RhoA in its active form is bound to GTP and undergoes a series of post-translational modifications of its C-terminal end that include isoprenylation, C-terminal proteolytic cleavage, and carboxymethylation in the endoplasmic reticulum (43). The processed RhoA is then translocated to the plasma membrane, where it binds to phosphatidylserine moieties and acts upon various effector molecules (46).

The cytoskeleton requirements in paramyxovirus infection have long been recognized (14, 15). However, to this point there has been no evidence of any viral protein involvement in the direct or indirect stimulation of actin filament reorganization. The cellular proteins involved in the interaction with RSV proteins and the nature of their interactions with the cytoskel-

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eton have not been defined. Since RSV F protein is important for RSV-induced syncytium formation, we attempted to identify F-interacting cellular proteins. A yeast two-hybrid screen was performed with RSV F as bait and a HeLa cDNA library as prey. We detected a small GTPase, RhoA, as an interacting partner of F. The interaction of F and RhoA was confirmed by various methods both in vivo and in vitro. The binding domains of F and RhoA were mapped. Further, we have shown that RhoA expression in the cell correlates with the number of RSV-induced plaques in cell culture.

MATERIALS AND METHODS

Virus and cells. The A2 strain of RSV was provided by R. Chanock, National Institutes of Health (NIH), Bethesda, Md. RSV stocks were prepared as previously described (21). HEp-2 cells were maintained in Eagle's minimal essential medium supplemented with glutamine, gentamicin, penicillin G, and 10% fetal bovine serum.

Yeast two-hybrid system. The extracellular domain of F gene was amplified by PCR and cloned into the *Eco*RI and *Bam*HI sites of the pAS2-BD (encodes Gal4 DNA-binding domain [BD]) vector (Clontech, Palo Alto, Calif.) such that a fusion between the Gal4 DNA-BD and the N terminus of the F gene is generated. Likewise, a HeLa cell cDNA library that had been constructed in the pGAD GH-AD (encodes Gal4 activation domain [AD]) vector to generate fusions between proteins encoded by the library cDNAs and the Gal4 AD was obtained from Clontech. The cotransformation and screening procedures were done as described in the manufacturer's protocol. Briefly, the two types of hybrid plasmids were cotransformed into *Saccharomyces cerevisiae* Y190 reporter host strain and the cotransformants expressing interacting proteins were selected on synthetic dropout media deficient in His, Leu, and Trp. To confirm the protein interaction, primary His+ transformants were tested for expression of the second reporter gene *lacZ* by using a β-galactosidase assay. All positive transformants were then retested to eliminate false positives.

Mammalian two-hybrid system. The extracellular domain of the F gene was cloned into the *Eco*RI and *Bam*HI sites of the pM vector (Clontech) to generate fusions of F protein with the Gal4 DNA-BD (named pM-F). Similarly, the RhoA gene was cloned into the *Eco*RI and *Xba*I sites of pVP16 (Clontech) to generate fusions of the protein RhoA with the VP16 AD (VP16 transcriptional activation domain, derived from the VP16 protein of the herpes simplex virus) (named pVP16-RhoA). A third vector, pG5CAT, provides a chloramphenicol acetyltransferase (CAT) reporter gene under control of a Gal4-responsive element and the minimal promoter of adenovirus E1b (Clontech). The three vectors were cotransfected into the HEp-2 human epithelial cell line by using Lipofectamine (Gibco BRL, Grand Island, N.Y.) by standard methods. pM-F and pVP16-RhoA were also transfected alone or in combination with the vectors pM and pVP16, to determine autonomous activation of the Gal4 reporter pG5CAT by the expression plasmids and also to determine the basal transcription potential of each plasmid. The interaction between the proteins F and RhoA was assayed by measuring CAT gene expression by using a CAT enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Indianapolis, Ind.). The level of CAT expression was determined by measuring the absorbance at 405 nm by using a microtiter plate reader (Dynatech, Chantilly, Va.).

ELISA. Immunoaffinity-purified RSV F glycoprotein (a gift from Wyeth-Lederle-Praxis Biologicals, West Henrietta, N.Y.) was diluted to 200 ng/ml in carbonate buffer (pH 9.6). One hundred microliters of F suspension was applied to wells of Immulon II 96-well plates (NUNC, Roskilde, Denmark). Blocking was performed with 3% bovine serum albumin and 3% nonfat dry milk for 1 h. One hundred microliters of RhoA or Rac1, another Rho family GTPase (CalBiochem, La Jolla, Calif.), at 200 ng/ml, was added separately and incubated at room temperature for 2 h, followed by addition of a 1:4,000 dilution of anti-RhoA or anti-Rac1 monoclonal antibodies (Santa Cruz Biotech, Santa Cruz, Calif.) after washing with phosphate-buffered saline–0.1% Tween 20. After 1 h, plates were washed and a 1:7,000 dilution of goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase was added. After washing, the substrate 3,3',5,5'-tetramethylbenzidine (Sigma, St. Louis, Mo.) was added and the color was read at 450 nm by using a microtiter plate reader. Immunoaffinity-purified RSV G glycoprotein (Wyeth-Lederle-Praxis Biologicals) was used instead of RSV F glycoprotein as a control.

BIA. Assays were designed according to the biomolecular interaction analysis (BIA) technology manual supplied by the manufacturer (Pharmacia Biosensor AB). Briefly, a capturing molecule, anti-F1 monoclonal antibody (kindly provided by Brian Murphy, NIH), was immobilized by amine coupling by using carbodiimide reaction, on the surface of a carboxymethylated dextran sensor chip. Immunoaffinity-purified F ligand was allowed to flow onto the surface of the immobilized monoclonal antibody so that the F protein was captured. Then, an analyte, RhoA protein, was allowed to flow onto the surface of immobilized ligand and the interaction was recorded on the sensorgram as resonance units (RU). Rac1 and the RSV surface glycoprotein G were used as negative analyte and ligand controls, respectively.

Coimmunoprecipitation. [^{35}S]methionine-labeled RSV stock was prepared as previously described (21) with modification. After 24 h postinfection, the cell monolayer was washed with methionine-free medium, incubated in this medium for 45 min, and then labeled with 200 μCi of [^{35}S]methionine (Amersham, Piscataway, N.J.) per ml of methionine-free medium for 24 h. For preparing RSV-infected and mock-infected cell lysates, HEp-2 cells were labeled with [^{35}S]methionine 2 h before addition of [^{35}S]methionine-labeled RSV and medium without RSV, respectively, and harvested 4 h after infection. Cells were lysed in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, and protease inhibitors), and insoluble material was pelleted by centrifugation at 16,000 × *g* for 5 min. Supernatants were incubated with 2 μg of each antibody overnight at 4°C. Immune complexes were bound to protein G-Sepharose beads (Sigma) for 2 h at 4°C, washed three times with lysis buffer, and eluted with 2× sample buffer (10 mM Tris-HCl [pH 6], 10% glycerol, 2% sodium dodecyl sulfate [SDS], 10% 2-mercaptoethanol, and 0.006% phenol red). The proteins were resolved by SDS–12% polyacrylamide gel electrophoresis. For reimmunoprecipitation of the eluted fraction, the elution from the first immune complex was combined with 20 μl of 10% SDS and heated at 95°C for 5 min. The eluted proteins were then dissolved in 700 μl of lysis buffer and incubated with the appropriate antibody followed by protein G-Sepharose beads. The second elution was done as described above.

Construction of RhoA protein deletion mutants. Clones encoding different RhoA deletion mutants were constructed by PCR amplification. All forward primers contained an *Eco*RI site, and all reverse primers carried an *Xho*I site. The respective forward and reverse primers were as follows: for $RhoA_{N32}$ amplification, 5'-GTCCCGGAATTCGATGGAGGTGTATGTGCCCACAG TGTTTG-3' and 5'-GCCGCTCGAGGCCAAGACAAGGCAACCAGA-3'; for $RhoA_{N67}$ amplification, 5'-GTCCCGGAATTCGATGGATCGCCTGAG GCCCCTCTCCTAC-3' and the same reverse primer as that for $RhoA_{N32}$; for $RhoA_{N110}$ amplification, 5'-GTCCCGGAATTCGATGGTGCCCATCATCCT GGTTGGGAATAAG-3' and the same reverse primer as that for $RhoA_{N32}$; for $RhoA_{C155}$ amplification, 5'-GTCCCGGAATTCGATGGCTGCCATCCGGAA GAAACTGGTG-3' and 5'-ATGCCGCTCGAGTCAGGTCTTTGCTGAACA CTCCATGTAC-3'; and for $RhoA_{N67\text{-}C110}$ amplification, the same forward primer as that for $RhoA_{N67}$ and 5'-CCGCCGCTCGAGGATGGGCACGTTGGGA CAGAAATGC-3'. PCR amplifications were carried out by using the pGAD GH-RhoA plasmid as a template and 30 cycles with steps of 1 min at 94°C, 1 min at 42°C, and 2 min at 72°C. PCR products were isolated and purified by agarose gel electrophoresis and were digested with *Eco*RI and *Xho*I. The resulting fragments were cloned into a pGAD GH vector that had been digested with *Eco*RI and *Xho*I. All constructs were sequenced by using a Sequenase sequencing kit (United States Biochemicals) to confirm that the correct bases were present. All primers were synthesized by IDT, Coralville, Iowa.

Construction of F protein deletion mutants. The F deletion mutants were constructed by PCR amplification. All forward primers contained an *Eco*RI site, and all reverse primers carried a *Bam*HI site. The respective forward and reverse primers were as follows: for F_{N550} amplification, 5'-GCAGCATGCCATGGAG TTGCTAATCCTCAAAGC-3' and 5'-GCGGCGCCTAGGATTTGTGGTGG ATTTACCAGC-3'; for F_{N137} amplification, 5'-GTCCCGGAATTCATGGGA TTTCTTGGTTTTTTGTTAGGTGTTGG-3' and the same reverse primer as that as for F_{N550}; for F_{N146} amplification, 5'-GTCCCGGAATTCATGTCTGC AATCGCCAGTGGCGTTGC-3' and the same reverse primer as that for F_{N550}; for F_{N155} amplification, 5'-GTCCCGGAATTCATGTCTAAGGTCCTGCACC TAGAAGGG-3' and the same reverse primer as that for F_{N550}; for F_{N224} amplification, 5'-GCATCGCGGATCCAATGTCAAATATAGAAACTGTG ATAGAGTTCC-3' and the same reverse primer as that for F_{N550}; for F_{N283} amplification, 5'-GGTAGGACTAGTTAGTGGTAATTGTACTACATATGC TAAG-3' and the same reverse primer as that for F_{N550}; and for $F_{N155\text{-}C467}$ amplification, 5'-GTCCCGGAATTCATGTCTAAGGTCCTGCACCTAGAA GGG-3' and 5'-GCGGCGCCTAGGTCATATTATTGGTTCACCTTTTACAT AGAG-3'. PGem7z-F plasmid containing the RSV F gene from strain A2 (gift from P. L. Collins, NIH) was used as a template and PCR amplified as described for RhoA constructs. The resulting fragments were cloned into the *Eco*RI and *Bam*HI sites of the pAS2-BD vector. All constructs were sequenced to confirm that the correct bases were present. For $F_{N146\text{-}C155}$, the following complementary oligonucleotides were synthesized with 5' extension of *Eco*RI and 3' extension of *Bam*HI sites: forward, 5'-AATTCTCTGCAATCGCCAGTGGCGTTGCTGTA TCTAAGGTG-3'; reverse, 5'-GATCCACCTTAGATACAGCAACGCCACT GGCGATTGCAGAG-3'. After annealing, the double-stranded $F_{N146\text{-}C155}$ was ligated in *Eco*RI- and *Bam*HI-digested pAS2-BD.

Ecdysone-inducible expression of RhoA. The Ecdysone-Inducible Expression system (Invitrogen, Carlsbad, Calif.) is based on the molting induction system found in *Drosophila* but is modified for inducible expression in mammalian cells. The system uses the steroid hormone ecdysone analog ponasterone A to activate expression of the gene of interest via a heterodimeric nuclear receptor. Ponasterone A has no detectable effect on mammalian cell physiology (36). Briefly, the human RhoA gene was PCR amplified by two external primers containing *Bam*HI and *Eco*RI restriction sites by using a plasmid containing the RhoA gene as a template. The amplified product obtained after restriction digestion was cloned into the *Bam*HI and *Eco*RI restriction sites of the pIND plasmid, which contains five modified ecdysone response elements upstream of a minimal heat shock promoter. The resulting construct, pIND-RhoA, was cotransfected with

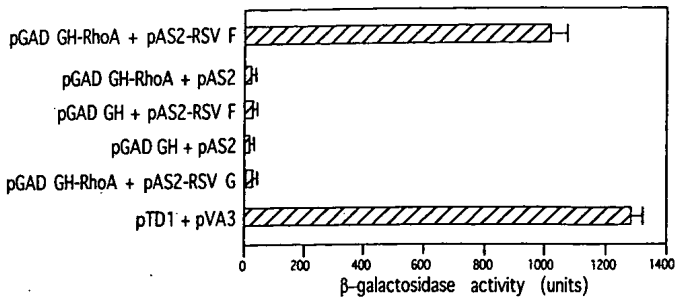

FIG. 1. RSV F interacts with RhoA in the yeast two-hybrid system. β-Galactosidase activity was quantified after yeast cells were cotransformed with the two indicated constructs. pAS2-RSV F and pAS2-RSV G contain the extracellular domains of RSV F and RSV G, respectively. pGAD GH-RhoA is RhoA-containing clone derived from the HeLa cell Matchmaker cDNA library. Cotransformation with pTD-1 (simian virus 40 large-T antigen) and pVA-3 (murine p53) is the positive control.

pVgRXR (which encodes the receptor subunits) into mammalian cells by using Lipofectamine (Gibco BRL) according to the manufacturer's protocol. After 48 h posttransfection, cells were split into fresh media containing Zeocin (300 μg/ml) and G418 (600 μg/ml).

Western blotting. After stable cell lines were established, intracellular RhoA expression was induced by treating the cells with ponasterone A for 24 h. HEp-2 cells and stably RhoA-transfected HEp-2 cells that were untreated with ponasterone A were used as controls for endogenous RhoA expression. RhoA expression was confirmed by Western blot analysis by using anti-RhoA antibodies. Cells were lysed in RIPA buffer, and insoluble material was pelleted by centrifugation at 16,000 × *g* for 5 min. Supernatants were mixed in 2× sample buffer, and the proteins were resolved on SDS–12% polyacrylamide gel. Separated proteins were transferred to a polyvinylidene difluoride membrane by standard methods. After blocking with 3% bovine serum albumin, a 1:2,000 dilution of anti-RhoA monoclonal antibodies was added, followed by addition of a 1:4,000 dilution of alkaline phosphatase-conjugated anti-mouse antibodies. A substrate, Fast Red TR/Naphthol AS (Sigma), was added and washed after the color development.

Plaque assay. Two-day-old HEp-2 cell monolayers, 80% confluent in 12-well plates (Costar, Cambridge, Mass.) were used for plaque assay (21). Intracellular RhoA expression was induced by treating the cells with ponasterone A for 24 h. HEp-2 cells and stably RhoA-transfected HEp-2 cells that were untreated with ponasterone A were used as controls. Twenty-four hours after treatment with ponasterone A, 100 μl of a solution containing RSV at 10^3 PFU/ml was added to HEp-2 cells in 12-well plates. After 3 days plates were fixed with 10% formalin and hematoxylin and eosin staining was performed. The number of RSV plaques in each well was determined.

RESULTS

RSV F-RhoA interaction in vivo. (i) Screening HeLa cell cDNA library in a yeast two-hybrid system. The yeast two-hybrid system was used to screen a HeLa cell cDNA library for encoded proteins capable of binding to a fusion protein containing RSV F protein. Of 3×10^6 clones screened from the library, one positive clone that had strong β-galactosidase activity, when the plasmid encoding RSV F protein and the plasmid encoding a protein from the HeLa cell library were coexpressed, was identified (Fig. 1). Comparison with the Swiss-Prot and Protein Data Bank databases indicated that the amino acid sequence was identical to that of RhoA. The pGAD GH-RhoA containing RhoA gene from the HeLa cDNA library had sequences from the RhoA 5′ noncoding region, a coding region that was in frame with the Gal4 transcriptional activation domain, and the 3′ noncoding region including a poly(A) tail. To demonstrate the specificity of the interaction, the identities of the bait and prey proteins were reversed such that the extracellular domain of RSV F protein was now expressed as a Gal4 AD-F fusion while RhoA protein was expressed as Gal4 BD fusion proteins. The results confirmed that there was an interaction between F and RhoA. Interaction was not detected when the RSV G protein was coexpressed with RhoA.

(ii) Mammalian two-hybrid system. We next asked whether the interaction of F with RhoA could be demonstrated in mammalian cells. To address this issue, we performed a mammalian two-hybrid analysis using transient transfection of the HEp-2 cells (Fig. 2). The extracellular domain of F fused to the Gal4 BD in the vector pM was cotransfected into HEp-2 cells with RhoA fused to the VP16 AD in the vector pVP16 and a reporter plasmid, pG5CAT. There was a significantly higher level (40-fold increase) of CAT expression in HEp-2 cell extracts expressing F and RhoA fusion proteins compared to the levels of CAT expression in HEp-2 cell extracts cotransfected with negative control plasmids. Thus, RSV F can interact with RhoA in vivo not only in yeast but also in mammalian cells.

(iii) Coimmunoprecipitation. To characterize further the in vivo association of RhoA and F, we examined whether the two proteins could be coimmunoprecipitated with anti-F1 monoclonal antibodies from RSV-infected or mock-infected HEp-2 cells (Fig. 3). HEp-2 cells were labeled with [^{35}S]methionine 2 h before addition of [^{35}S]methionine-labeled RSV and harvested 4 h after infection. RSV-infected cell lysates immunoprecipitated with anti-F1 monoclonal antibody (lane 1) showed F0, F1, F2, and RhoA proteins. Of the eluted precipitate from lane 1, 75% was reimmunoprecipitated with anti-RhoA antibodies (lane 2) and showed RhoA protein. Cell lysates from mock-infected HEp-2 cells immunoprecipitated with anti-F1 monoclonal antibody (lane 3) showed no F protein but when immunoprecipitated with anti-RhoA antibodies (lane 4) showed RhoA protein. Immunoprecipitation of mock-infected cell lysates with anti-F1 antibodies followed by reimmunoprecipitation of the eluted fraction with anti-RhoA antibodies did not produce F0, F1, F2, or RhoA protein (lane 5). In vitro-translated RhoA is shown in lane 6. Mouse isotype control was also used to coimmunoprecipitate RSV-infected cells. However, no protein with a molecular weight corresponding to that of F0, F1, F2, or RhoA protein was seen on the gel (data not shown). The data demonstrate that RhoA associates with the RSV F protein, thus confirming the interaction of RhoA and F in an RSV-infected mammalian cell. These data collectively demonstrate the interaction of RSV F with RhoA in vivo.

RSV F-RhoA interaction in vitro. Since the in vivo experiments did not reveal whether F interacts with RhoA-GTP or RhoA-GDP, we examined whether RSV F interacts with a RhoA-GDP form in vitro by ELISA and by BIA. For in vitro assays, we used immunoaffinity-purified full-length F protein

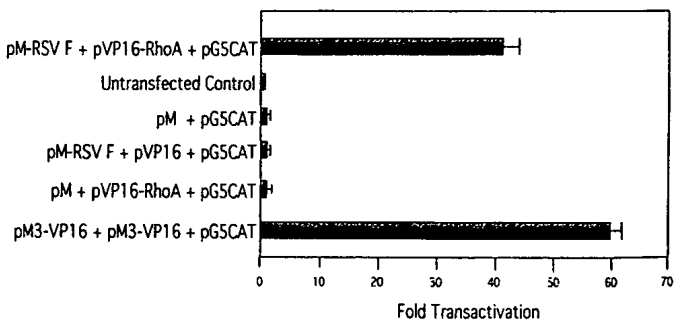

FIG. 2. F-RhoA interaction analysis in the mammalian two-hybrid system. The level of CAT enzyme activity detected in a culture of HEp-2 cells transfected with pM and pG5CAT reporter plasmid was set at 1.0. Levels of CAT expression observed when HEp-2 cultures were additionally cotransfected with the indicated VP16 fusion protein expression plasmids are given as multiples of this value. Cotransfection with pM3-VP16 (encoding a fusion of the Gal4 DNA-BD and the VP16 AD) and pG5CAT is the positive control.

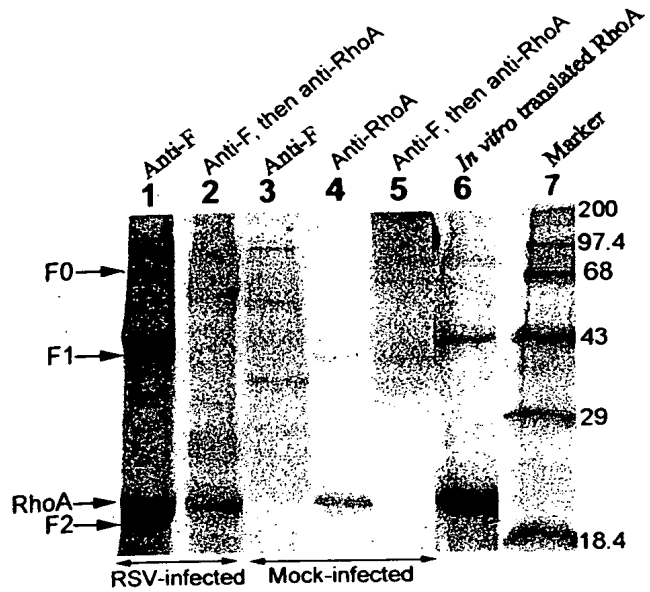

FIG. 3. Coimmunoprecipitation of RSV F and RhoA. HEp-2 cells were labeled with [^{35}S]methionine beginning 2 h before infection of [^{35}S]methionine-labeled RSV and continuing until harvest at 4 h after infection. Proteins were analyzed on SDS–12% polyacrylamide gel. Lane 1, protein lysates isolated from RSV-infected HEp-2 cells were immunoprecipitated with an anti-F1 monoclonal antibody; lane 2, 75% of the anti-F1 immunoprecipitated lysate was reimmunoprecipitated with anti-RhoA polyclonal antibodies; lane 3, protein lysates isolated from mock-infected HEp-2 cells were immunoprecipitated with an anti-F1 monoclonal antibody; lane 4, protein lysates isolated from mock-infected HEp-2 cells were immunoprecipitated with anti-RhoA polyclonal antibodies; lane 5, protein lysates isolated from mock-infected HEp-2 cells were immunoprecipitated with an anti-F1 monoclonal antibody and the eluted fraction was reimmunoprecipitated with anti-RhoA polyclonal antibodies; lane 6, in vitro-translated RhoA protein expressed by using the TNT rabbit reticulocyte lysate system (Promega); lane 7, marker (positions of molecular size markers are shown on the right and expressed in kilodaltons). Positions of RSV F0, F1, F2, and RhoA proteins are indicated at the left.

derived from RSV and purified recombinant RhoA protein or Rac1 protein expressed in *Escherichia coli* (Calbiochem).

(i) ELISA. Purified RSV F (20 ng/well) was applied to wells of Immulon II 96-well plates. After blocking, 20 ng of either RhoA or Rac1 was added separately to each well and the binding to F protein was detected by anti-RhoA or anti-Rac1 monoclonal antibodies. RSV F and RhoA interaction resulted in a 60-fold increase in absorbance compared to those of controls in which either recombinant Rac1 protein was added instead of RhoA or immunoaffinity-purified RSV surface glycoprotein G was added instead of RSV F protein (Fig. 4). This indicates that RSV F can bind RhoA-GDP and also confirms the interaction of both proteins in vitro.

(ii) BIA. To characterize further in vitro association of purified RSV F and RhoA-GDP, we examined the interaction of both proteins by real-time BIA (Fig. 5) using the BIAcore 2000 instrument. F protein was captured by anti-F1 monoclonal antibodies immobilized on the surface of the carboxymethylated dextran sensor chip. RhoA was allowed to flow onto the surface of immobilized F, and the interaction was recorded on the sensorgram as resonance units. As controls, Rac1 protein was used instead of RhoA and RSV surface glycoprotein G was used instead of RSV F protein. F-RhoA interaction gave a response of 976 RU (corresponding to binding of approximately 0.97 ng of RhoA to F per mm^2 on the sensor chip surface) above the baseline RU. Experiments in which RSV G was used as a ligand control and Rac1 protein was used as an analyte control gave values similar to their respective baseline

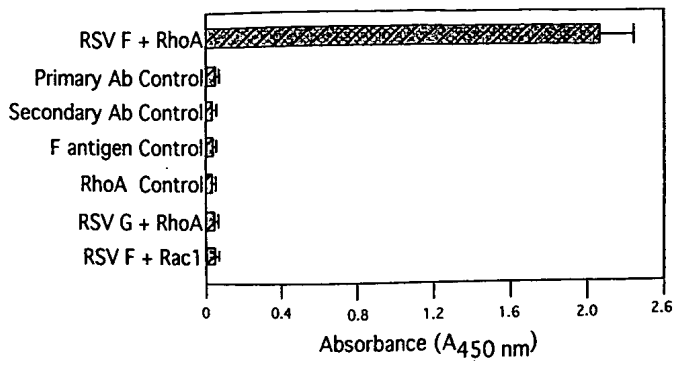

FIG. 4. F-RhoA interaction analysis by ELISA. Wells were coated with 20 ng of immunoaffinity-purified RSV F protein followed by addition of RhoA. Bound RhoA was detected by ELISA by using anti-RhoA monoclonal antibody and by measuring absorbance at 450 nm. Rac1 was used instead of RhoA as a negative control, and bound Rac1 was detected by anti-Rac1 antibody. RSV G glycoprotein was used instead of RSV F glycoprotein as a control.

RU (phase a to b). These data support the ability of RSV F to interact with RhoA-GDP in vitro.

Mapping of the binding domain of RSV F. In order to map the binding domain of F protein, we constructed N-terminal and C-terminal deletion mutants of F protein by PCR amplification methods and studied the interactions in a yeast two-hybrid system (Fig. 6). Yeast transformants coexpressing F_{N550} or F_{N137} and RhoA proteins gave positive blue color as measured by β-galactosidase assay. A yeast transformant designated F_{N146} having an N-terminal nine-amino-acid deletion from F1 fusion peptide gave intense blue color, and colonies grew more rapidly and larger than F_{N137}, indicating strong interaction with the RhoA protein. This also suggests that deleting the hydrophobic amino terminus may have resulted in a conformational change in the F1 protein which increased interaction with RhoA. Alternatively, the F_{N146} constructed without part of the fusion domain may be less toxic than F_{N137} to the yeast cells and allow more rapid growth. Yeast transformants with F_{N155}, F_{N224}, F_{N283}, and $F_{N155\text{-}C467}$ deletion

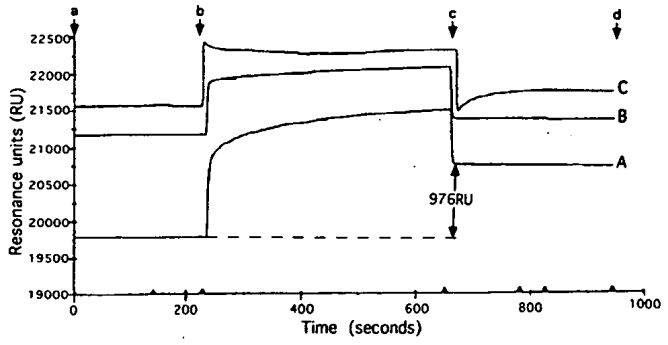

FIG. 5. F-RhoA interaction analysis by real-time BIA using the BIAcore 2000 instrument. Immunoaffinity-purified F ligand was captured by immobilized anti-F1 monoclonal antibody on the surface of the carboxymethylated dextran sensor chip. An analyte, RhoA, was allowed to flow on the surface of F ligand, and the interaction was recorded on the sensorgram as RU. The RU at baseline (phase a to b) corresponds to the amount of capture ligand immobilized on the sensor chip. Phase b to c corresponds to the injection of analyte. Phase c to d corresponds to the injection of buffer to wash nonspecifically bound analyte molecules. 976RU corresponds to binding of approximately 0.97 ng of RhoA to F per mm^2 on the sensor chip surface (A). RSV G (B) and Rac1 (C) proteins were used as negative ligand and analyte controls, respectively. The dashed line has been added to more easily show the deflection above baseline when RhoA interacts with F.

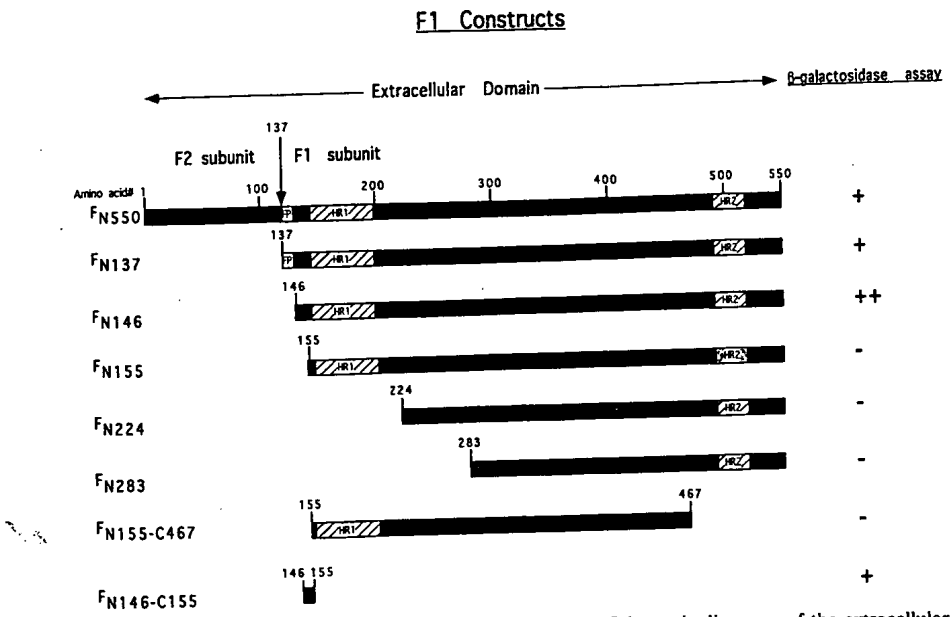

FIG. 6. Mapping of the binding domain of RSV F protein in a yeast two-hybrid system. Schematic diagrams of the extracellular domain of RSV F protein and different truncation mutants are shown. The amino acid sequence numbers of wild-type F are shown at the top. The cleavage site of F0, F1, and F2 subunits and positions of fusion peptide (FP) and heptad repeats (HR1 and HR2) are shown on the F protein. The subscripts of F mutant names indicate deletions of sequences from either the N- or C-terminal end of F. pAS2 plasmid containing F mutant was cotransformed with pGAD GH-RhoA plasmid in yeast, and the interaction was detected by β-galactosidase assay. + and ++, relative intensities of blue color of the colonies after X-Gal staining; −, no blue colonies observed after X-Gal staining.

mutant proteins did not interact with the RhoA protein as measured by β-galactosidase assay. These results suggest that the RhoA binding domain in RSV F is contained within or overlaps the region between amino acids 146 and 155 of the F protein. We then constructed $F_{N146\text{-}C155}$, encoding the amino acids (146 to 155) which showed interaction with RhoA protein. The interaction suggests that the RhoA binding domain is contained within this nine-amino-acid region of RSV F.

Mapping of the binding domain of RhoA. Next, the RhoA binding domain was mapped (Fig. 7A). We constructed N-terminal and C-terminal deletion mutants of RhoA protein by PCR amplification methods and studied the interactions in a yeast two-hybrid system. The yeast transformants with pAS2-F_{N550} and pGAD GH plasmids encoding various deletion mutants, designated $RhoA_{N32}$, $RhoA_{N67}$, and $RhoA_{C155}$, gave blue color in a β-galactosidase assay, suggesting that binding to F was not affected. However, yeast transformant colonies with pAS2-F_{N550} and plasmid encoding $RhoA_{N110}$ did not grow, indicating that the F protein did not bind to RhoA protein and that the binding site had been deleted. This result suggests that the F binding domain lies between amino acids 67 and 110 of RhoA. The interaction of this binding domain with F was confirmed by β-galactosidase assay by using the sequence encoding $RhoA_{N67\text{-}C110}$.

RhoA facilitates RSV-induced syncytium formation. We next correlated RhoA expression in HEp-2 cells with RSV-induced syncytium formation. This was carried out using a stably transfected HEp-2 cell line in which RhoA was expressed from an ecdysone-inducible promoter (Fig. 8). There was some leaky expression of RhoA in uninduced stably transfected HEp-2 cells (lane 2) compared to the levels of RhoA in normal HEp-2 cells (lane 1) (Fig. 8A). To study the effect of RhoA overexpression on RSV infection, the induction of RhoA expression was initiated 24 h before RSV infection with the ecdysone analog, ponasterone A, and continued for 48 h after infection. RhoA expression correlated with the number (Fig. 8B) and size (data not shown) of RSV-induced plaques in each cell line. There were statistically significant differences between plaque numbers for induced, RhoA-transfected cells and those for uninduced, RhoA-transfected cells and normal HEp-2 cells ($P < 0.001$ and $P < 0.0001$, respectively; two-tailed t test). Syncytium formation could be seen as early as 20 h after infection of induced, RhoA-transfected cells, whereas no syncytia were seen in ponasterone A-treated normal HEp-2 cells until after 48 h. These results demonstrate that upregulation of intracellular RhoA expression facilitates RSV-induced syncytium formation.

DISCUSSION

Our report is the first to describe a viral protein interacting with RhoA and also the first to describe a cellular ligand found to interact with an RSV protein. We have shown that RhoA interacts with RSV F both in vivo by yeast two-hybrid assay, mammalian two-hybrid assay, and coimmunoprecipitation and in vitro by ELISA and BIAcore. We have mapped the binding domains of RSV F and RhoA which may represent potential targets for the development of novel antiviral therapy. In addition, we have shown that RhoA expression correlates with the formation of RSV plaques.

Since viral surface glycoproteins with transmembrane regions are not transported to the nucleus, where the protein-protein interaction occurs in the yeast two-hybrid system, we used the extracellular domain of F to screen the HeLa cell cDNA library. This was confirmed by using a full-length F construct in a yeast two-hybrid screening in addition to the extracellular domain of F. The full-length F protein did not interact with any of the HeLa cDNA proteins as no yeast colonies grew on the selective media. The mapping studies of F and RhoA further confirmed that the extracellular domain of F is transported to the nucleus for interaction with RhoA in the yeast two-hybrid system.

We used a mammalian two-hybrid assay to confirm the interaction of F with RhoA. Because the assay is performed in

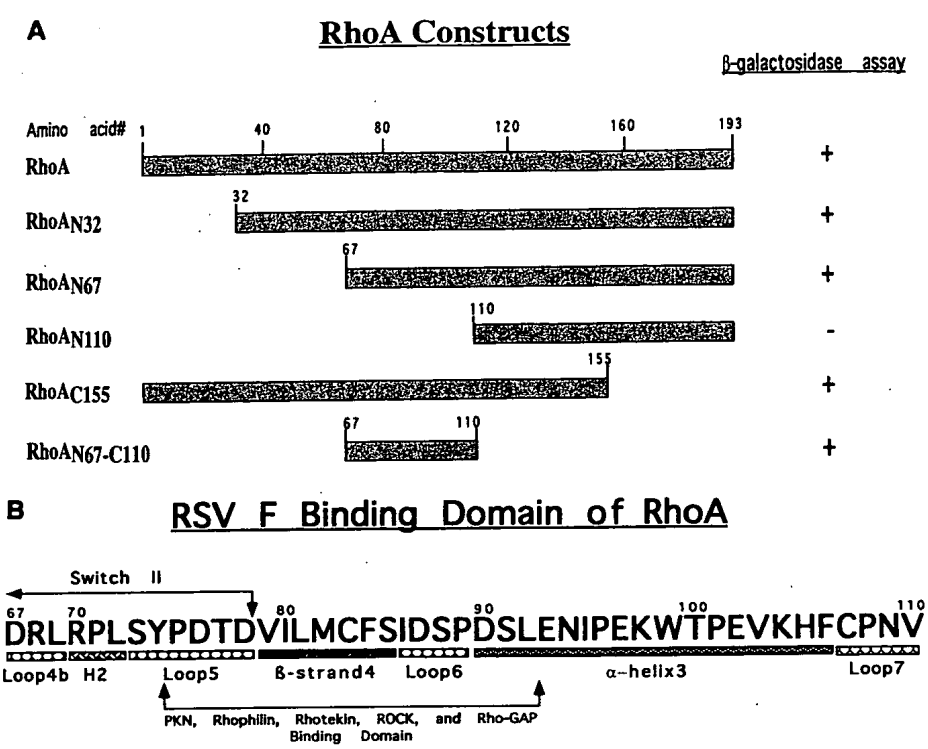

FIG. 7. (A) Mapping of the binding domain of RhoA protein in a yeast two-hybrid system. Schematic diagrams of the full-length RhoA protein and different truncation mutants are shown. The amino acid sequence numbers of wild-type RhoA are shown at the top. The subscripts of RhoA mutant names indicate deletions of sequences from either the N- or C-terminal end of RhoA. pGAD GH plasmid containing RhoA mutant was cotransformed with pAS2-F plasmid in yeast, and the interaction was detected by β-galactosidase assay. +, blue colonies detected after X-Gal staining; −, no blue colonies detected after X-Gal staining. (B) Amino acid sequence of RSV F binding domain of RhoA showing binding of RhoA effector molecules. The positions of amino acid residues of RhoA are indicated above the sequence. The bars indicate the positions of loop 4b, 3_{10}-helix (H2), loop 5, extended β-strand 4, loop 6, α-helix 3, and loop 7. Part of the switch II region is shown. The binding regions of PKN and PRK2 (serine/threonine protein kinases), rhophilin, rhoketin, Rho kinases (ROCK), and Rho-GAP are shown.

mammalian cells, interactions between the proteins are more likely to be biologically significant. The results obtained in the mammalian two-hybrid assay showing a 40-fold increase in the CAT expression levels indicate that the RSV F can interact with RhoA in vivo in mammalian cells. They also support the probability that the interactions between the proteins are authentic and that the folding of binding domains of both proteins in yeast is similar to that in mammalian cells.

Unlike in yeast or mammalian two-hybrid systems, where interaction occurs in the nucleus, the site of interaction of F and RhoA in RSV-infected cells resembles natural infection, and the interaction of both the proteins is more authentic. Since RhoA is endogenously expressed in HEp-2 cells and to characterize further the in vivo association of F and RhoA in a native condition, we examined whether the two proteins could be coimmunoprecipitated with anti-F1 monoclonal antibodies from mock-infected and RSV-infected HEp-2 cells. Coimmunoprecipitation revealed that the interaction of F with RhoA occurs in RSV-infected HEp-2 cells; this is significant evidence of a direct association of the molecules during the process of infection. It is possible that RSV F and RhoA interaction may have occurred after detergent lysis of the cells. To address this concern, we mixed detergent-lysed RSV-infected cells with in vitro-translated RhoA, but no interaction was observed. These data suggest that after the F protein is treated with detergent and boiled, it is unable to interact with RhoA.

RhoA cycles between two states, i.e., an active, GTP-bound form and an inactive, GDP-bound form (3). In its inactive state, RhoA localizes to the cytoplasm in a complex with RhoA-GDP dissociation inhibitor (GDI) but translocates to the plasma membrane upon activation (46). As shown by crystallographic studies of RhoA, the structure of RhoA bound to GTP reveals a fold similar to that of RhoA-GDP but shows conformational differences localized in switch I (amino acids 28 to 38) and switch II (amino acids 61 to 78) (25, 52). The locus of binding of GTP or GDP to RhoA is in a phosphate-binding loop (amino acids 13 to 20) and the switch I region. The in vivo data from the yeast two-hybrid assay, the mammalian two-hybrid assay, and coimmunoprecipitation do not reveal whether RhoA is in the GDP- or GTP-bound form. Therefore, in order to determine whether F can bind RhoA in its GDP-bound form and whether RhoA-GDP can alter the structure of the F binding domain, we carried out in vitro binding experiments using recombinant RhoA. The results from ELISA indicate that F can bind RhoA-GDP and further suggest that the structure of the F binding domain in RhoA-GDP may not be affected. Although GTP or GDP binding to RhoA may not be a prerequisite for the association of F with RhoA in vitro, GTP binding to RhoA may be necessary for biological functions of the F and RhoA complex in vivo. It will therefore be important in future studies to define whether F binds equally well to Rho-GTP and whether this has relevance for downstream signaling events that can be mediated by RhoA. Although a number of mitogens, namely, lysophosphatidic acid, growth factors, and thrombin, are known to activate RhoA, an upstream ligand for RhoA has not been identified. The RSV F protein may therefore have value as a reagent in future studies of RhoA activation and signal transduction.

In ELISA, the F protein was immobilized by adsorption to

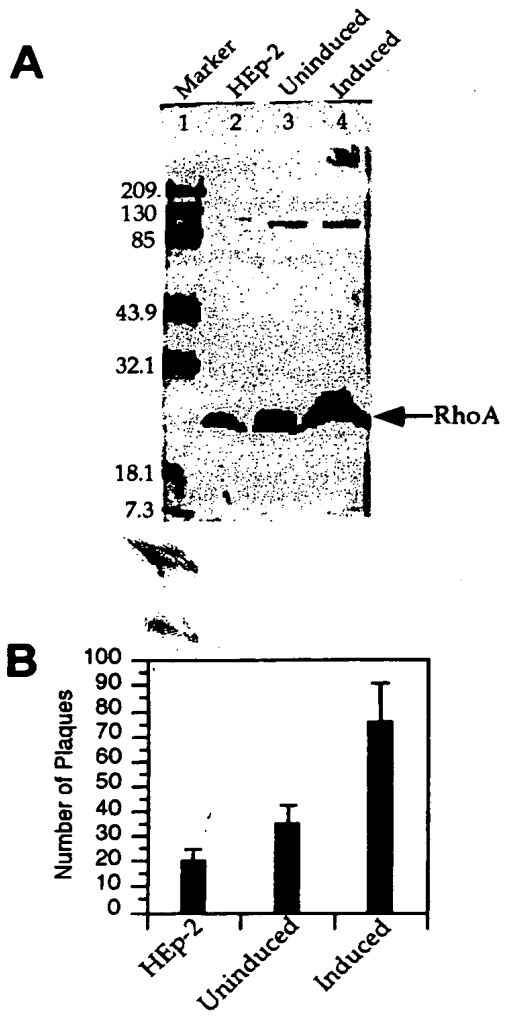

FIG. 8. RhoA promotes RSV-induced syncytium formation. (A) RhoA expression in stably RhoA-transfected and normal HEp-2 cells analyzed by Western blotting. Stably RhoA-transfected HEp-2 cells were either uninduced or induced for RhoA expression by the ecdysone analog ponasterone A. Normal HEp-2 cells without added ponasterone A were used as a control for endogenous RhoA expression. Twenty-four hours after induction, the cells were lysed and the proteins were resolved on an SDS–12% polyacrylamide gel. The proteins were transferred to a polyvinylidene difluoride membrane by standard methods. RhoA expression from ecdysone-induced (lane 4) and uninduced (lane 3) stably RhoA-transfected HEp-2 cells and from normal HEp-2 (lane 2) cells were analyzed by Western blotting by using anti-RhoA polyclonal antibodies. Positions of molecular size markers (lane 1) are shown on the left and expressed in kilodaltons. (B) Correlation of RhoA expression and RSV-induced plaque formation. RSV stock (100 μl) was added to either normal HEp-2 cells or uninduced or induced RhoA-expressing stably transfected HEp-2 cells in 12-well plates. RhoA expression was induced 24 h prior to RSV infection. After 2 days, plates were fixed with methanol and RSV-specific immunoperoxidase staining was performed. The numbers of plaques were determined and are shown as arithmetic means ± standard deviations. Two independent experiments were performed, each in duplicate.

plastic and this may at least partially alter the conformation of the protein. To address this concern and to further confirm the in vitro association of F and RhoA, we used the BIA, which is based on the surface plasmon resonance phenomenon. This method makes it possible to visualize the binding process as a function of time by monitoring the increase in refractive index that occurs when RhoA interacts with F that is captured by immobilized anti-F1 monoclonal antibody on the surface of a sensor chip. The native conformation of the immobilized F protein may be better preserved since it is bound to anti-F1 antibody rather than directly to the chip. The other advantage is that none of the proteins needs to be labeled or conjugated, which avoids the artifactual changes in binding properties that often result when proteins are labeled or conjugated (49). The results from BIA suggest that F binds RhoA with strong affinity and that the dissociation of bound RhoA is very slow in comparison to that of controls. The density of RhoA bound to F was approximately 0.97 ng/mm^2 on the sensor chip surface, which is significant compared to controls. This high-affinity interaction between F and RhoA may be essential to the integrity and stability of the complex in biological systems to compete with host proteins for this domain of RhoA.

Mapping of the binding domains of F and RhoA has made it possible to determine critical domains of both proteins involved in the biological functions that may unravel new pathways involved in the replication of RSV. In mapping the domain for F in a yeast two-hybrid system, a weak interaction of RhoA with F_{N137} was seen, in contrast to a strong interaction of RhoA with F_{N146} deletion mutants. This suggests that there may be conformational determinants that affect access to the RhoA binding domain, thereby preventing RhoA interaction. These data also suggest that a conformational change or unfolding of fusion peptide may have to occur prior to or during RhoA binding. The binding domain (amino acids 146 to 154) is present within a part of the fusion peptide (amino acids 137 to 154), indicating that this region may be important for events involving binding with RhoA leading to virus entry. It is also possible that by stimulating RhoA, the various biological functions of RhoA, such as actin bundling, may be utilized for cell-to-cell spread by syncytium formation, virus assembly, and maturation. Since RSV infection in cell culture can occur despite the lack of the putative G and SH proteins (27), it is not surprising that F may have additional unknown functions. It is well known that F is involved in virus entry and syncytium formation, but it is possible that F may also be involved indirectly in virus maturation. For example, RhoA activation promotes reorganization of actin which could potentially serve as scaffolding in the formation of filamentous RSV particles.

It has been well established that the key molecular determinants for RhoA-effector protein binding are the switch I and switch II domains (17). Recently, a determinant for effector binding located between RhoA residues 75 and 92 was identified (53). The crystal structure of RhoA (25, 41, 42) indicates that the F protein binding domain in RhoA between amino acids 67 and 110 is a groove on the molecule bounded by a helical coil. This suggests that the binding domain in F could extend into the RhoA "pocket." This region of RhoA is important for interaction of various downstream effectors (Fig. 7B) that regulate multiple cellular processes. This region of RhoA was previously shown to bind a Rho GTPase-activating protein (GAP) (18, 41, 42), suggesting the potential for F binding to either increase or decrease GTPase activation during virus infection. Loop 6 (amino acids 87 to 90) of RhoA has been shown to bind two classes of effector kinases, represented by PKN or PRK2 (serine/threonine protein kinases) and Rho kinases (ROKα/ROCK-II/Rho-kinase and ROKβ/ROCK-I/p160ROCK), that mediate Rho-induced stress fiber formation and cellular transformation (53). In addition, loop 6 also binds two nonkinase molecules, rhophilin and rhotekin (17). It is possible that the F protein by interacting with RhoA may be simulating or blocking one of the effector functions mediated by RhoA. Alternatively, binding of this region of RhoA may confer conformational changes in F. There is considerable evidence that RhoA induces a rapid reorganization of actin into stress fibers in a variety of cell lines (39). In RSV infection, there is a stress fiber formation early in the infection (20). The

stimulation of RhoA by F may lead to actin reorganization leading to stress fiber formation.

Previous work has shown that in RSV infection actin filaments are necessary for transcription (24), syncytium formation, and virus maturation (5, 19). In the last few years, actin has also been shown to play important roles in gene transcription, syncytium formation, and maturation of many other viruses (4, 11, 44, 50). Precise temporal and spatial control of actin filament organization is essential for these activities, but how this organization is achieved is not known. The interaction of viral protein with RhoA may have significance in virus infection to ensure a coordinated control of cellular activities required for virus replication, such as determination of the stage of the cell cycle and cytokinesis during syncytium formation and virus maturation. Although RhoA is ubiquitously expressed in all tissues, lung tissue expresses RhoA at a very high level (16). This may be one of the explanations for an efficient replication of RSV in lung tissue. This hypothesis is supported by the increased number (Fig. 8B) and size of RSV plaques and the speed of their formation (data not shown) when RhoA is overexpressed in stably RhoA-transfected HEp-2 cells and clearly indicates physiological significance of RhoA in RSV infection. It is possible that increased RhoA expression enhances the number of RSV plaques by facilitating virus entry and cell-to-cell spread. Alternatively, increased RhoA activity may alter the cytoskeleton structure to indirectly improve the efficiency of virus infection. It is also possible that efficient virus replication and maturation may be affected by RhoA influences on gene transcription, rapid actin bundling, and regulation of cell morphology at the level of virus assembly.

Although the interaction of RSV F and RhoA is surprising, given its wide range of biological functions essential for survival of the cell and possibly for the virus to replicate within a short time, it is not surprising for a virus to target such a key molecule. In RSV infection of A549 cells (an airway epithelial cell line) and primary bronchial cells, there are increased levels of interleukin-8 (2, 37) and NF-κB (31), in addition to actin reorganization (5, 19) and stress fiber formation (20). Previous studies have shown that thrombin increases RSV-induced syncytium formation (13) and that inhibitors of thrombin inhibit RSV-induced syncytium formation in cell culture (47). RhoA expression is increased by treatment with thrombin (35), and activation of RhoA results in the increased levels of NF-κB (38, 40) and bradykinin (38) and actin reorganization and stress fiber formation (22, 35). RhoA is critical in actomyosin-based contractility as it increases calcium sensitivity in smooth muscles (23). Indeed RhoA activation has been shown to promote myosin kinase activity which induces bronchiolar smooth muscle contraction (28) and has been suggested to play a role in asthma (45). It is therefore intriguing to consider RhoA activation as a possible step in the process of RSV-induced wheezing caused by smooth muscle contraction. Thus, biological effects of RhoA activation add a new dimension to RSV pathogenesis.

Although our data support the involvement of RhoA during RSV infection, it is unclear where in the cell RSV F binds to RhoA. Therefore, additional work is needed to investigate not only the precise locus of interaction of F and RhoA but also the downstream signaling events potentially triggered by RhoA interaction with the RSV F protein during virus infection. Since many enveloped viruses have similar needs for utilizing actin for various steps in the virus life cycle, a common mechanism involving RhoA GTPase may be shared among some viruses of different families. The binding domain of F and RhoA may be an important target for developing novel therapy and designing a better vaccine for RSV. RhoA-derived peptides from the F binding domain or other molecules that interfere with the F and RhoA interaction may provide a novel therapeutic approach. One could also envision that an appropriate mutation in the RhoA binding region of F may attenuate live recombinant RSV to produce a candidate vaccine.

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